ESTABLISHMENT AND MAINTENANCE OF CNIDARIAN – DINOFLAGELLATE SYMBIOSES

by

DANIEL J. THORNHILL

(Under the Direction of William K. Fitt)

ABSTRACT

Dinoflagellates of the genus *Symbiodinium* form symbioses with a variety of animal and protistan hosts, including reef-building corals. Growing concern about the global degradation of coral reefs has underscored the need to understand these complex symbiotic relationships. This dissertation documents the extent to which corals and other symbiotic cnidarians show fluctuations in their associations with *Symbiodinium* spp.

The presence of free-living *Symbiodinium* and the onset of symbiosis in juvenile hosts were investigated using aposymbiotic scyphistomae of the jellyfish *Cassiopea xamachana*. Scyphistomae exposed to various habitats took a variety of distinctive symbionts into symbiosis including members of sub-generic clades A, B, C and D. Adult *C. xamachana* possessed only *Symbiodinium* type A1 implying that specificity likely develops as a result of post-infection processes due to competitive exclusion or other mechanisms.

The genetic identity of *Symbiodinium* was examined seasonally in six species of broadcast spawning corals (*Acropora palmata*, *A. cervicornis*, *Siderastrea siderea*, *Montastrea faveolata*, *M. annularis*, and *M. franksi*) from the Bahamas and Florida Keys. Most coral colonies showed little to no change in their dominant symbiont. However, certain colonies of *M. annularis* and *M. franksi* from the Florida Keys exhibited significant shifts in their associations attributed to recovery from the stresses of the 1997-98 El Niño Southern Oscillation (ENSO) event. Intracolonial shifts of mixed *Symbiodinium* populations were also examined for colonies of *M. annularis* from Florida.

To test whether mode of symbiont acquisition influences the stability of coral-dinoflagellate symbioses, three species of brooding coral species (*Agaricia agaricites*, *Porites astreoides*, and *S. radians*) were examined seasonally for several years. Symbiotic associations in all brooding colonies did not vary, suggesting that reproductive mode has a significant effect on the stability of these symbioses.

Finally, the physiology of the host-symbiont association (holobiont) was examined in colonies of *M. annularis* which harboring different *Symbiodinium* communities. Colonies harboring populations of mixed symbiont types, including the putatively stress tolerant type D1a *Symbiodinium*, had significantly different zooxanthellae densities, photosynthetic capacity of PSII and host tissue biomass than colonies harboring exclusively type B1. After type D1a was

displaced, no significant differences were detected, suggesting that the identity of *Symbiodinium* harbored significantly effects the physiology of the holobiont.

INDEX WORDS: Caribbean, coral bleaching, symbiont acquisition, symbiosis, zooxanthellae, *Acropora*, *Agaricia*, *Cassiopea, Montastrea*, *Porites*, *Siderastrea, Symbiodinium*

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CHAPTER 1

INTRODUCTION

Symbiodinium, the genus of dinoflagellate that associates with cnidarians, is genetically and physiologically diverse (Rowan and Powers 1991; Trench 1993, 1997; Rowan 1998; LaJeunesse 2001). Once believed to be a single species (Freudenthal 1962; Taylor 1974), the genus is now recognized as eight genetically divergent sub-generic clades (A-H) (reviewed by Baker 2003). Each clade comprises numerous "types" that are distinguishable genetically, ecologically, and biogeographically (LaJeunesse 2001; 2002; LaJeunesse et al. 2003. 2004a). Among cultured *Symbiodinium* spp., broad differences in morphology and physiology have been observed (Schoenberg and Trench 1980a,b,c; Chang et al. 1983; Colley and Trench 1983; Fitt 1984, 1985a; Iglesias-Prieto and Trench 1994, 1997; Warner et al. 1999; Kinzie et al. 2001).

The mechanism of symbiont acquisition depends on the reproductive strategy of the coral host (Richmond and Hunter 1990). Most corals broadcast spawn azooxanthellate gametes into the water column (Szmant 1986; Richmond and Hunter 1990). In such cases symbionts must be 'horizontally' acquired from the surrounding environment, thereby creating the possibility for new host-symbiont combinations with each new generation (Szmant 1986; Richmond and Hunter 1990). In contrast corals that internally brooded their larvae transfer symbionts 'vertically' from the maternal parent to offspring (Szmant 1986; Richmond and Hunter 1990). Because of the maternal transmission of zooxanthellae, symbioses in brooding corals are hypothesized to be more specific than those of broadcast spawners (e.g. Loh et al. 2001). Most

available data supports this hypothesis. For instance, Loh et al. (2001) found a higher degree of regional divergence in the symbionts of the brooding *Seriatopora hystrix* compared to the broadcasting *Acropora longicyathus* suggesting regional host-symbiont coevolution and specialization in brooding corals. Across a wide biogeographic gradient, LaJeunesse et al. (2004b) found environment and latitude determined symbiont association in broadcast spawning corals whereas brooding corals associated with a number of closely related symbionts specialized exclusively on a single host genera. Similarly, Barneah et al. (2004) found that symbiont clade correlated with mode of acquisition among Red Sea soft corals. However, other studies found that mode of symbiont acquisition does not affect the overall level of symbiont diversity in *Porites* spp. (Hidaka and Hirose 2000) or Indo-Pacific Acroporid corals (van Oppen, 2004) suggesting that brooding corals are more open to exogenous symbiont infection than previous hypothesized.

Previous research has demonstrated that many aposymbiotic cnidarians are capable of infection with multiple types of symbiotic algae, often exceeding the symbiotic diversity found in adult hosts. For instance, numerous forms of *Chlorella* spp. are readily phagocytosed by freshwater *Hydra viridis* (e.g. McAuley and Smith, 1982; Rahat and Sugiyama, 1993). Similar patterns are well documented for the initial uptake of various *Symbiodinium* spp. in scyphistomae (polyp-stage) of the jellyfish *Cassiopea xamachana* (Colley and Trench 1983; Fitt 1984; Fitt 1985a), sea anemones (Kinzie and Chee 1979; Schoenberg and Trench 1980; Davy et al. 1997; Belda-Ballie et al. 2002), tridacnid clams *Hippopus hippopus* (Fitt 1985a), gorgonians (Kinzie 1974; Benayahu et al., 1989; Coffroth et al. 2001), and scleractinian corals (Schwartz et al. 1999; Weis et al. 2001; Little et al. 2004; Rodriguez-Lanetty et al. 2004). However, following phagocytosis of *Symbiodinium* spp., most of these studies show symbiont specificity early in the

ontogeny of the host, with preference for homologous algae (those originating from same host) over heterologous algae (those originating from a different host). Homologous algae have superior growth rates and competitive ability in many hosts (e.g. Fitt 1985a, Belda-Ballie et al. 2002). The recent availability of molecular methods to investigate *Symbiodinium* diversity and associations has provided additional information on the development of host-symbiont specificity. Coffroth et al. (2001) documented non-specific uptake of *Symbiodinium* spp. by aposymbiotic juvenile gorgonians, however, specificity was eventually achieved, as clade B *Symbiodinium* became the only detectable symbiont lineage. A similar pattern occurred in another gorgonian species (Santos et al. 2003). Comparisons of three different *Symbiodinium* ITS types of clade C in the scleractinian coral *Fungia scutaria* demonstrated specific selection of symbionts both during and after infection (Rodriguez-Lanetty et al., 2004).

Symbiodinium spp. are continuously released from their hosts (e.g. Steele 1976, Hoegh-Guldberg et al. 1987, Stimson and Kinzie 1991), yet little is known about the actual abundance or diversity of *Symbiodinium* living in the external environment. Free-living dinoflagellates matching the description of *Symbiodinium* have occasionally been found and cultured from the external environment (e.g. Loeblich and Sherley 1979; Carlos et al. 1999; LaJeunesse 2001; Schwarz et al. 2002). Field infection experiments utilizing aposymbiotic gorgonian hosts indicate that viable symbiont cells are readily available from the surrounding water (Coffroth et al. 2001). Longer exposure time to waters of the reef ecosystem and/or to dilute solutions containing cultured *Symbiodinium* spp. in the laboratory increased the percentage of animals infected. The rate of spread of a symbiont population within host tissues appears to also depend on the initial dose received during the exposure/treatment phase (Coffroth et al., 2001).

Most coral colonies possess a monotypic *Symbiodinium* spp. population (Rowan 1998; Baker 1999; van Oppen et al. 2001; LaJeunesse 2002; Goulet and Coffroth 2003; LaJeunesse et al. 2003, 2004a; Santos et al. 2004). However, host species often show variation with more than one symbiont throughout their geographic and depth distributions, although specific combinations are highly dependent on the species of coral (LaJeunesse 2002, LaJeunesse et al. 2003, LaJeunesse et al. 2004a,b), their specific environment (Rowan et al. 1998) and their geographic location (Rodriguez-Lanetty et al. 2001, Loh et al. 2001, LaJeunesse 2002, LaJeunesse et al. 2003, LaJeunesse et al. 2004a,b, LaJeunesse 2005). Additionally, some coral species may harbor multiple clade types within a single colony (Baker and Rowan 1997; Ulstrup and van Oppen 2003; Chen et al. 2005). Occasionally, mixed symbiont populations are found in colonies from intermediate or shallow reef habitats. The distribution of different symbionts within a single colony correlates with irradiance levels and may relate ultimately to differences in photophysiology among symbionts (Rowan et al. 1997). Manipulation of coral colonies containing more than one type of *Symbiodinium* can result in re-assortment of symbiont types to regions of the colony exposed to altered light conditions (Rowan et al. 1997; Toller et al. 2001). Few mixed symbiont populations have ever been observed in deep, low light environments (LaJeunesse 2002).

Coral reefs represent one of the most sensitive ecosystems on the planet. Over the past two decades, episodes of catastrophic bleaching and mortality have occurred with increasing intensity, in response to warm, calm, sea-surface conditions. The severity of these global events has lead to dire predictions about the future persistence of coral reefs in face of increasing global warming (Glynn 1991, Hoegh-Guldburg 1999, Hughes et al. 2003). Growing knowledge of the host-symbiont compositions of corals has caused conjecture about how they become modified in

response to climate and/or environmental change (Buddemeier and Fautin 1993; Baker 2001; LaJeunesse 2005). The modern coral reef ecosystem has ancient origins, emerging in the early Cenozoic (Wood 1998). Reefs have persisted through dramatic shifts in temperature (Haq et al. 1987), yet extant corals are paradoxically sensitive to slight temperature increases of 1-2 ˚C above normal summer highs (Hoegh-Guldberg 1999; Hughes et al. 2003). Hypothetically, a change in the dinoflagellate symbiont to one better adapted to the prevailing environment could optimize the physiology of the holobiont association (Rowan and Powers 1991; Buddemeier and Fautin 1993).

While it has been widely hypothesized that the lineage of symbiont significantly impacts the physiology of the host-symbiont combination (holobiont) (Buddemeier and Fautin 1993; LaJeunesse 2005), relatively few studies have addressed this question directly. For instance, Little et al. (2004) showed that symbiont type had a highly significant effect on the colony growth rate in two species of *Acropora* from the Great Barrier Reef (GBR); colonies harboring clade C *Symbiodinium* grew two to three times faster than those harboring clade D. Thermal tolerance is of particular interest because of growing concern over the temperature and light induced bleaching (reviewed in Lesser 2004). Thermally tolerant *Symbiodinium* spp. are known from several clades (Tchernov et al. 2004). LaJeunesse et al. (2003) found that bleaching-resistant *Montipora digitata* from the GBR harbored ITS type C15 Symbiodinium, whereas thermally sensitive *Montipora* spp. harbored other C types. Clade D is widely reported as imparting thermal tolerance because of its presence in colonies from high temperature environments (Toller et al. 2001a; van Oppen et al. 2001) as well as in colonies recovering from bleaching (Toller et al. 2001b; Baker et al. 2004). Rowan (2004) showed that *Pocillopora* spp. corals harboring *Symbiodinium* clade D maintained or even improved their photosynthetic

capacity of PSII and maximum net photosynthesis when subjected to increasing temperature. Similarly treated *Pocillopora* spp. harboring clade C were temperature sensitive, experiencing chronic photoinhibition and decreases in maximum net photosynthetic rate (Rowan 2004).

In most coral species, the dominant *Symbiodinium* spp. type does not appear to change seasonally (Goulet and Coffroth 2003; Thornhill et al. 2005; but see Chen et al. 2005). However, scleractinian corals and their symbiotic dinoflagellates (zooxanthellae) do experience significant seasonal fluctuations in many physiological parameters. For instance, zooxanthellae undergo regular seasonal changes in density, a phenomenon believed to be ubiquitous among symbiotic corals (Muller-Parker 1987; Stimson 1997; Brown et al. 1999; Fagoonee et al. 1999; Fitt et al. 2000). Seasonal highs in zooxanthellae density typically occur during the winter months when temperature and irradiance are at the lowest annual levels (Fagoonee et al. 1999; Fitt et al. 2000). Similar fluctuations occur in the pigment content (chlorophyll-a) (Brown 1999; Fitt et al. 2000; Warner et al. 2002) and photosynthetic capacity of PSII (Warner et al. 2002), with circannual maxima occurring during the winter months. Coral tissue biomass (as measured by ash-free dry weight) likewise changes seasonally (Fitt et al. 2000; Warner et al. 2002). However peak tissue biomass occurs in the spring after peaks in zooxanthellae density, suggesting that that there is a functional relationship between these two parameters (Fitt et al. 2000). Corals living in shallow water usually have higher levels and more significant fluctuations of all these parameters (except chlorophyll-a) (Fitt et al. 2000). Nothing is known about how these patterns change when a host associates with different types of *Symbiodinium* spp.

This dissertation examines how symbioses between cnidarians and *Symbiodinium* are established and maintained. Chapter 2 examines how symbioses are established in a model system, the schyphistomae (polyp stage) of *Cassiopea xamachana*. I show how the initial

openness to multiple lineages of symbionts is followed by early ontogenic establishment of specificity. Through use of this experimental model I also make inferences about the availability of free-living *Symbiodinium* spp. Chapters 3 and 5 demonstrated how symbioses are maintained overtime in scleractinian corals, in response to seasonal fluctuations and following a major coral bleaching event in 1997-1998. These two chapters examine how reproductive mode effects symbiont specificity and stability. Chapter 3 looks at this phenomenon in five species of broadcast spawning corals (*Acropora palmata*, *A. cervicornis*, *Siderastrea siderea*, *Montastrea faveolata*, *M. annularis*, and *M. franksi*), which are predicted to be more flexible in their symbioses than the three species of brooding corals (*Agaricia agaricites*, *Porites astreoides*, and *S. radians*), examined in Chapter 5. Chapter 4 examines intracolonial patterns in symbioses from the highly variable *Montastrea annularis* species complex (Lopex et al. 1999). Having examined how symbioses change over time, Chapter 6 uses a natural experiment of changing symbioses in *M. annularis* to test the influence of symbiont type on the physiology of the holobiont. Chapter 6 summarizes and discusses the information presented in this dissertation.

Four appendices provide additional information on projects which I have been involved in throughout my graduate studies. Appendix A is a study of *Symbiodinium* biogeography from the corals and other symbiotic invertebrates of the Hawaiian Islands. We report higher levels of symbiont specificity among Hawaiian corals than those from reefs of the Western Atlantic or Great Barrier Reef. Appendix B is an experiment that tests the holosymbiont pigment response to thermal and light stress. Appendix C reports the utility of *C. xamachana* as a bioindicator species. This bioindicator is then used to measure the nutrient flux on reefs of the Upper Florida Keys at a sensitivity greatly exceeding conventional methods of measurement. Finally Appendix D examines the effect of parrotfish grazing on symbioses in *M. annularis*, showing that

chronically grazed corals have lower zooxanthellae densities and greater symbiont diversity than ungrazed colonies.

CHAPTER 2

PATTERNS OF INFECTION BY FREE-LIVING *SYMBIODINIUM* POPULATIONS IN APOSYMBIOTIC SCYPHISTOMAE OF THE JELLYFISH *CASSIOPEA XAMACHANA*[1]

[1] Thornhill DJ, Daniel MW, LaJeunesse TC, Fitt WK, and GW Schmidt, to be submitted to the *Journal of Experimental Marine Biology and Ecology*.

Introduction

Symbiotic cnidarians often harbor only a single genotype of symbiotic dinoflagellates in the genus *Symbiodinium* (e.g. LaJeunesse, 2002; Goulet and Coffroth, 2003; LaJeunesse et al., 2003; Santos et al., 2003; Thornhill et al. *in press*). However, many host species are capable of symbiosis with more than one *Symbiodinium* spp. type; these different symbiotic associations are typically partitioned by the external physical environment or geographic location (i.e. irradiance, depth and temperature related to latitude) (Rowan and Knowlton 1995; Rodriguez-Lanetty et al. 2001; van Oppen et al. 2001; LaJeunesse 2002; LaJeunesse et al. 2004a,b).

Partner recombination, where an alternate symbiont population replaces the resident population, is a hypothetical mechanism by which symbiotic cnidarians adjust to changes in their surrounding environment (Buddemeier and Fautin, 1993; LaJeunesse 2005). The larvae of most broadcast spawning corals, many species of Rhizostome scyphozoans, and numerous other cnidarians depend on horizontal acquisition from environmental pools of *Symbiodinium* to establish their symbioses (Fitt 1984; Szmant 1986; Richmond 1997). In contrast, brooded larvae usually acquire their symbionts from their maternal parent prior to their release, a process referred to as vertical transmission (Richmond 1997). Previous work on horizontal acquisition indicates that chemical cues play a role in bringing symbionts and hosts together (Fitt 1985b) and that entry and persistence of healthy symbionts somehow blocks fusion of lysosomes with the phagosome (Hohman et al. 1982; Fitt and Trench, 1983). Specificity in these "open" systems generally appears to be less stringent and corals exhibiting such broadcast spawning life histories tend to show greater variability in associating with particular symbionts over environmental and geographic gradients (Rowan et al., 1997; LaJeunesse et al., 2004b).

Despite the constancy and stability observed for many adult coral-algal symbioses (Goulet and Coffroth 2003; Stat et al. 2004; Thornhill et al., *in press*), experiments have demonstrated that many aposymbiotic cnidarians are capable of infection with multiple types of symbiotic algae, often exceeding the symbiotic diversity found in adult hosts. For instance, numerous forms of *Chlorella* spp. are readily phagocytosed by freshwater *Hydra viridis* (e.g. McAuley and Smith, 1982; Rahat and Sugiyama, 1993). Similar patterns are well documented for the initial uptake of various *Symbiodinium* spp. in scyphistomae (polyp-stage) of the jellyfish *Cassiopea xamachana* (Colley and Trench 1983; Fitt 1984; Fitt 1985a), sea anemones (Kinzie and Chee 1979; Schoenberg and Trench 1980; Davy et al. 1997; Belda-Ballie et al. 2002), tridacnid clams *Hippopus hippopus* (Fitt 1985a), gorgonians (Kinzie 1974; Benayahu et al., 1989; Coffroth et al. 2001), and scleractinian corals (Schwartz et al. 1999; Weis et al. 2001; Little et al. 2004; Rodriguez-Lanetty et al. 2004). However, following phagocytosis of *Symbiodinium* spp., most of these studies show symbiont specificity early in the ontogeny of the host, with preference for homologous algae (those originating from same host) over heterologous algae (those originating from a different host). Homologous algae have superior growth rates and competitive ability in many hosts (e.g. Fitt 1985a, Belda-Ballie et al. 2002). The recent availability of molecular methods to investigate *Symbiodinium* diversity and associations has provided additional information on the development of host-symbiont specificity. Coffroth et al. (2001) documented non-specific uptake of *Symbiodinium* spp. by aposymbiotic juvenile gorgonians, however, specificity was eventually achieved, as clade B *Symbiodinium* became the only detectable symbiont lineage. A similar pattern occurred in another gorgonian species (Santos et al. 2003). Comparisons of three different *Symbiodinium* ITS types of clade C in the

scleractinian coral *Fungia scutaria* demonstrated specific selection of symbionts both during and after infection (Rodriguez-Lanetty et al., 2004).

Symbiodinium spp. are continuously released from their hosts (e.g. Steele 1976, Hoegh-Guldberg et al. 1987, Stimson and Kinzie 1991), yet little is known about the actual abundance or diversity of *Symbiodinium* living in the external environment. Free-living dinoflagellates matching the description of *Symbiodinium* have occasionally been found and cultured from the external environment (e.g. Loeblich and Sherley 1979; Carlos et al. 1999; LaJeunesse 2001; Schwarz et al. 2002). Field infection experiments utilizing aposymbiotic gorgonian hosts indicate that viable symbiont cells are readily available from the surrounding water (Coffroth et al. 2001). Longer exposure time to waters of the reef ecosystem and/or to dilute solutions containing cultured *Symbiodinium* spp. in the laboratory increased the percentage of animals infected. The rate of spread of a symbiont population within host tissues appears to also depend on the initial dose received during the exposure/treatment phase (Coffroth et al., 2001).

The initial lack of specificity by aposymbiotic scyphistomae of *C. xamachana* for a particular *Symbiodinium* spp. during acquisition (Colley and Trench 1983; Fitt 1984; 1985a) provides a mechanism to investigate the abundance and diversity of free-living pools of *Symbiodinium*. The current study investigates infection from environmental pools of *Symbiodinium* spp. off Key Largo, Florida. We document temporal aspects of infection and identify various types of *Symbiodinium* taken into symbiosis with scyphistomae of *C. xamachana.*

Methods

Prior to this experiment, one clonal line of azooxanthellate scyphistomae of *Cassiopea xamachana* was grown and maintained in clean Petri dishes containing 35ppt Instant Ocean™ in a 26°C incubator with 100 μmol photons/m^2 ·sec illumination at under 14 hours of illumination per day. Animals were fed *Artemia* nauplii twice weekly and the water changed after each feeding.

In March of 2003, schyphistomae were placed in 10 ml centrifuge tubes with modified, open ends covered by 100 μm mesh plankton netting. These tubes were placed in 1-liter Nalgene bottles containing 0.45 μm-filtered seawater for transportation to various habitats in the waters around Key Largo, Florida. Twelve replicate tubes, each containing 3-5 aposymbiotic scyphistomae, were placed on Admiral Patch Reef off Key Largo. Six tubes were stationed on six colonies (one tube per colony) of the scleractinian coral *Montastrea faveolata* and six other tubes were tethered to a three cement bricks (two tubes per brick) in a small sand patch surrounded by a diversity of symbiotic anthozoans and octocorals containing various *Symbiodinium* ITS types (LaJeunesse 2002). Additional, similarly prepared, tubes of scyphistomae were placed in Florida Bay, with four tubes each next a colony (one tube per colony) of the coral *Siderastrea radians*, four tubes next to the anemone *Aiptasia palida* (each tube surrounded by a separate cluster of anemones), four tubes attached to four different heads of the symbiotic sponge *Anthosigmella varians*, and four tubes tethered to a four cement bricks (one tube per brick) in beds of *Thalasia* sea grass. Half of the tubes at each location were collected after 3 days of exposure, the other half after 5 days. The tubes were placed in 1-liter Nalgene bottles and transported to the laboratory where the polyps were rinsed with 0.45 μm-filtered seawater into clean Petri dishes (one dish per polyp) and placed in a 26°C incubator with 100

μmol photons/m^2 ·sec illumination. Animals were fed *Artemia* nauplii twice weekly and the water changed after each feeding. Mortality rates were low (<5%) for all locations. Control scyphistomae (n = 20) were maintained under similar conditions, but without field exposure. Colley and Trench (1983) found that unsuccessful strains of zooxanthellae rapidly declined in density to undetectable levels after four to twelve days. Therefore, the presence of zooxanthellae was determined visually using a microscope 21 days post exposure for all scyphistomae, to ensure that only successful associations were measured. To test the hypothesis that length of exposure had an effect zooxanthellae infection rate, data were analyzed by a paired t test for independence by groups ($\alpha = 0.05$) with the analytical software Statistica™ (Statsoft, 1999).

Scyphistomae were similarly exposed during May, August, and November of 2003, except that exposure time was 5 days during each season and tubes of scyphistomae were placed only in Florida Bay next to the same species as in the March samples. To test the hypothesis that time of year has an effect zooxanthellae infection rate, data were analyzed using one-way ANOVA ($\alpha = 0.05$) with the analytical software Statistica™ (Statsoft, 1999). A planned comparison least-significant-difference test was used for pair-wise comparisons between seasons.

Symbiodinium were identified from the scyphistomae and strobilated ephyra collected in March 2003 after three to five months of laboratory maintenance when densities of symbionts were in the 10,000-50,000 per polyp range, as estimated from previous hemocytometer counts of infected poylps. Nucleic acids were extracted using the Wizard DNA preparation protocol (Promega). Symbiotic individuals were macerated with a 2-ml tissue grinder in 600 μl of nuclei lysis buffer (Promega). The sample was transferred into a 2 ml centrifuge tube containing 200 μl of 0.5mm glass beads, then was bead beaten for 120 s at 4.8×10^3 rpm in a Biospec Mini-

Beadbeater. 2.5 µl of 0.1mg ml^{-1} Proteinase K was added and the sample was incubated at 65 ºC for 1hr, followed by incubation at 37 ºC for 30 min with 3 µl of 100 mg/ml Raze A. 250 µl of Protein Precipitation Solution (Promega) was mixed with each sample and samples were placed on ice for 30 min. The precipitate was pelleted by centrifugation for 10 min at 1.3 $x10^4$ x *g*. 600 µl of supernatant nucleic acids were precipitated by transfer to a new 1.5 ml microcentrifuge tube containing 700 µl 100% isopropanol and 50 µl of 3M NaAc (pH 5.6) and incubated on ice for a minimum of 15 min. The tube was then centrifuged for 10 min at 1.3 $x10^4$ x g, the supernatant removed, and the pellet washed with 70% ethanol. The DNA was centrifuged again for 5 minutes, dried and resuspended in 95 µl of water and 5 µl of 400 mM Tris, 10 mM –EDTA solution.

The internal transcribed spacer 2 region (ITS 2) of nuclear ribosomal RNA was used to discriminate molecular types of *Symbiodinium* (LaJeunesse, 2001; LaJeunesse, 2002). This region was amplified from 0.5-3 µl of DNA extract for denaturing-gradient gel electrophoresis (DGGE) using primers "ITS 2 clamp" and "ITSintfor 2" (LaJeunesse and Trench, 2000). PCR amplification followed the "touchdown" thermal cycle profile described by LaJeunesse (LaJeunesse, 2002). Products of these PCR reactions were checked by electrophoresis on agarose gels (0.8% agarose in 40 mM Tris-acetate, 1mM EDTA solution). Successfully amplified PCR products were subsequently electrophoresed in denaturing gradient gels (45-80% formamide, 8% acrylamide denaturing gradient gels; 100% consists of 7 *M* urea and 40% deionized formamide) following the protocol described by LaJeunesse and Trench (2000), with the modifications of LaJeunesse et al. (2003).

Results

Virtually all clades of *Symbiodinium* known to reside in cnidarians (clades A, B, C, and D) were identified from symbiotic scyphistomae and ephyra cultured from March of 2003, including ITS2 types A1, B1, C3, and D1a (Table 2.1). A sample diagnostic PCR-DGGE profile is show for all types detected in this study (Figure 2.1). Adult C. xamachana medusae scyphozoans from Florida Bay were found exclusively with *Symbiodinium* type A1 (Table 2.1, LaJeunesse 2002). Additionally, nearest neighbor host symbiont type was identified for those scyphistomae immediately adjacent to another symbiotic organism (Table 2.1). There was no apparent relationship between where the tubes were placed and which type of *Symbiodinium* was taken into symbiosis (Table 2.1).

Percentages of scyphistomae containing zooxanthellae 21 days following a 5-day field exposure were 50% in March 2003 (n=17 out of 34 samples), and increased to 100% in May (n=54), August (n=36) and November 2003 (n=36) (Fig. D.2.1). A one-way analysis of variance (ANOVA) revealed infectivity rates differed as a function of season ($F(3,156) = 40.95$, $p < .001$) with infectivity rates in March 2003 being significantly lower than that of all other groups ($p < .001$) (Fig. D.2.2).

In March, a higher percentage of polyps were infected when exposed to field conditions 5 days compared to 3 days on both Florida Bay and Admiral Patch Reef. However, this difference between 3 and 5 day exposures was only significant on Admiral Reef ($p < .05$). About twice the percentage of animals were infected in Florida Bay after 3 days compared to those placed on the patch reef (Figure 2.3), and this difference was significant ($p < .05$). No scyphistomae from the control group were infected during the experiment (n=20).

Discussion

The results of this study demonstrate the capability of aposymbiotic *Cassiopea xamachana* to be infected by multiple types of symbiotic algae in the field. This corroborates the results of laboratory studies where *C. xamachana* formed successful symbioses with a number of *Symbiodinium* spp. (Schoenberg and Trench 1980, Colley and Trench 1983, Fitt 1985). Many aposymbiotic cnidarian hosts similarly phagocytose a range of symbiotic algae (often with limited selectivity), however specificity for homologous algae is often apparent soon after infection with homologous algae exhibiting superior growth rates and competitive ability (Kinzie 1974; Kinzie and Chee 1979; Schoenberg and Trench 1980; McAuley and Smith, 1982; Colley and Trench 1983; Fitt and Trench 1983; Fitt 1984; Fitt 1985a; Benayahu et al., 1989; Rahat and Sugiyama, 1993; Davy et al. 1997; Coffroth et al. 2001; van Oppen et al. 2001; Weis et al. 2001; Belda-Ballie et al. 2002; Rodriguez-Lanetty et al. 2004). In the experiments detailed here, scyphistomae of *C. xamachana* retained representatives of every major clade of *Symbiodinium* found in association with scleractinian corals, including clades A, B, C, and D (Table 2.1). Since adult *Cassiopea* medusae were detected exclusively in symbiosis with ITS type A1 symbionts in Florida (LaJeunesse, 2002; Table 2.1), selection for A1 in *C. xamachana* occurs post-phagocytosis (see also Coffroth et al. 2001; Rodriguez-Lanetty et al. 2004), possibly by differential growth rates and competition within the host gastrodermal cell (e.g. Fitt; 1985, Belda-Baillie et al. 2002) or by some other, currently unknown mechanism.

There was no apparent relationship between where the scyphistomae were placed on the reef (nearest neighbor symbiont type) and which type of *Symbiodinium* was taken into symbiosis (Table 2.1). For instance, most scyphistoma placed near the coral *Montastrea faveolata* contained D1a, a type of symbiont never found in those coral colonies during this experiment

(Table 2.1) or through five years of seasonal sampling (Thornhill et al. *in press*, Appendix A), suggesting that the D1a symbionts originated from environmental pools, such as free-living *Symbiodinium* (Carlos et al., 1999), or was released from another nearby host (e.g. Reimer, 1971; Steele, 1977; Lee et al., 1995). Alternatively the symbionts detected in experimental schyphistomae could originate from their nearest host at detection levels below the ≥7% threshold of DGGE (Thornhill et al. *in press*). Similarly, in Florida Bay, *Symbiodinium* type C3 was identified from scyphistomae positioned near the anemone *Aiptasia tagetes*, and the sponge *Anthosigmella varians*, which contained B1 and B11 respectively. *Symbiodinium* A1 and C3 were identified from polyps adjacent to colonies the coral *Siderastrea radians*, which harbored a specialist symbiont, type B5a only found in the genus *Siderastrea*.

The four types of *Symbiodinium* detected in *C. xamachana* during this study are just a subset of types present in these environments (see LaJeunesse 2002). Previous work in the laboratory also demonstrated that *C. xamachana* while capable of symbioses with numerous strains of algae, did not form symbioses with all available strains (Schoenberg and Trench 1980, Colley and Trench 1983, Fitt 1985). This selectivity may be a result of zooxanthellae size (Fitt 1985), competitive ability (Fitt 1985; Belda-Ballie et al. 2002), or some other unknown mechanism (host symbiont recognition mechanisms, etc.). Similar results have been documented for other hosts that form symbioses with multiple lineages of *Symbiodinium* (e.g. Davy et al. 1997; Belda-Ballie et al. 2002; Thornhill et al. in press). The success of several *Symbiodinium* types in *C. xamachana*, is one example of a flexible symbiosis in the spectrum of cnidarian – algal symbioses which exist (reviewed in Baker 2003).

Our experimental approach may have been additionally biased by culturing time required to obtain sufficient material for DGGE analysis. Scyphistomae were maintained for three to five

months following exposure to reef water. During this time, the artificial conditions likely select for certain symbiont types to the exclusion of others. As a result, we cannot assume that the *Symbiodinium* types detected were the only symbionts to become infected during the 3 to 5 day exposure. Despite this limitation, it is still apparent that a diversity of *Symbiodinium* types (clades A-D) were readily available from the water column and were capable of producing viable symbioses in *C. xamachana*.

Laboratory experiments show that infection rate is correlated to algal concentration (Montgomery and Kremer, 1995; Coffroth et al., 2001; Kinzie et al., 2001) and time of exposure (Figure 2, but not Figure 3 in Coffroth et al., 2001). Differences in infection rate vs. exposure time in the current experiments (Figure 2.2) suggest that these same principles are acting in nature. Coffroth et al. (2001) found 100% infection of octocoral polyps placed in the field in Panama for 3-7 days, with time-to-visible infection much greater than that seen in laboratory experiments, where all polyps exposed to 500/ml density of *Symbiodinium* for 2-9 days became visibly infected within 2-3 weeks. Backreef exposure took a shorter time course similar to that seen in laboratory experiments, implying higher densities of potential symbiont availability, whereas animals placed on the fore-reef and deep reef took 2-3 weeks longer to look visibly infected (ibid). The data presented in Figure 2.2 show significantly lower infection rates of scyphistomae during the coldest season (March), suggesting that availability of free-living symbionts changes seasonally. Alternatively, scyphistomae may be less able to establish symbioses at lower temperatures. In addition, 3-day exposure infection levels were significantly lower on the reef (~15%) compared to scyphistomae placed in Florida Bay (~33%), potentially indicating spatial variability of availability of free-living *Symbiodinium.* Differences in uptake rates in bay and patch-reef environments, as well as lower infection rates during the coldest

season, suggest that availability of potential symbionts varies both temporally and spatially (Figs. 2.2 & 2.3). Variations in the availability of symbiont pools may influence post-bleaching re-infection processes thereby affecting the ability of corals and other symbiotic cnidarians to survive periods of physiological stress (see Lewis and Coffroth, 2004).

Table 2.1 *Symbiodinium* ITS2 types found in *Cassiopea xamachana* following exposure seawater of Florida Bay and Admiral Reef off of Key Largo, Florida in March 2003. *Symbiodinium* types were identified by DGGE analysis from scyphistomae and newly strobilated medusae (ephyra) and are listed using the alphanumeric nomenclature of LaJeunesse (2001). Parentheses following nearest-host denotes the symbiont type found in that host. Adult medusae of *C. xamachana* (n=6) collected from Florida Bay in March 2003 all contain the symbiont identified as type A1.

Holobiont	Nearest host	Location	*n*	ITS2 Type
Scyphistomae				
	Aiptasia tagetes (A4)	Fl. Bay	3	C3
	Anthostigmella (B11)	Fl. Bay	3	C3
	Siderastrea radians (B5A)	Fl. Bay	2	A1
	Siderastrea radians (B5A)	Fl. Bay	1	C3
	None, seagrass	Fl. Bay	3	C3
	None, seagrass	Fl. Bay	1	A1
	Montastrea faveolata (B1)	Adm. Reef	4	D1a
	None, reef	Adm. Reef	6	B1
Ephyra				
	Aiptasia tagetes (A4)	Fl. Bay	1	C3
	Montastrea faveolata (B1)	Adm. Reef	1	D1a
	None, seagrass	Fl. Bay	1	C3
Adult Medusae	-	Fl. Bay	6	A1

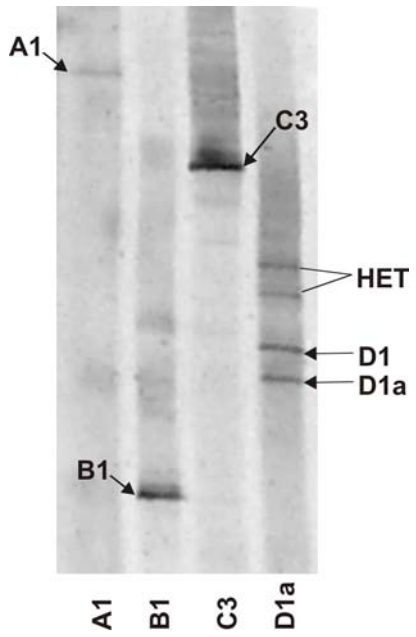


Fig. 2.1 Example PCR-DGGE profile of the *Symbiodinium* ITS 2 region for all *Symbiodinium* types detected in symbiosis with *Cassiopea xamachana* schyphistomae. Diagnostic band labeled for types A1, B1, C3 and D1a. Faint, repeated bands in each profile are possibly rare intragenomic variants and heteroduplexes (HET) created between them and dominant sequences.

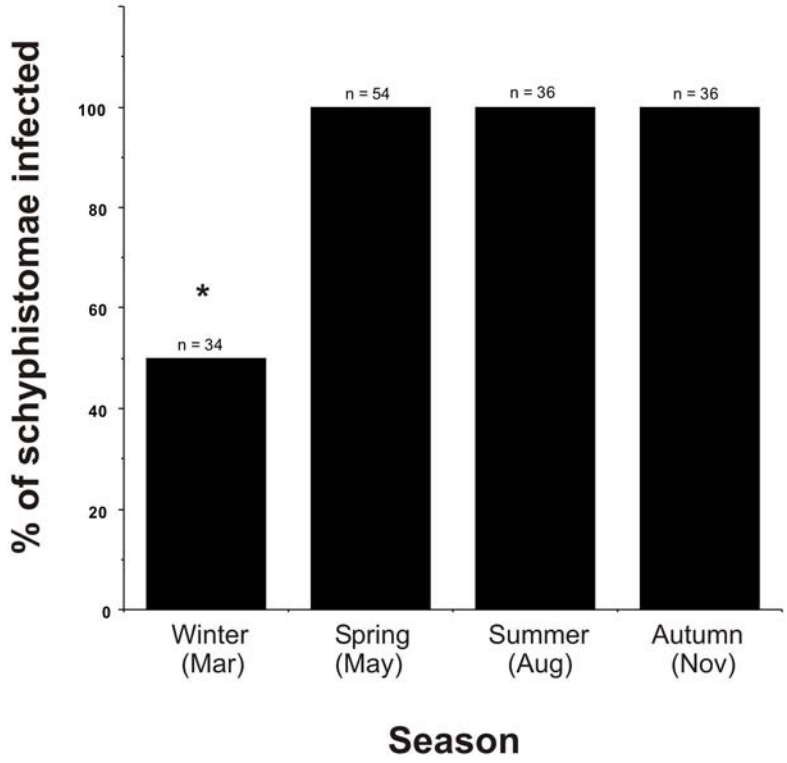


Figure 2.2 Percentage of scyphistomae of *Cassiopea xamachana* containing zooxanthellae following a 5-day exposure in Florida Bay through seasons of 2003. The number of samples (number of polyps) is listed above each histogram. * denotes a significant effect of season when compared to all other treatments.

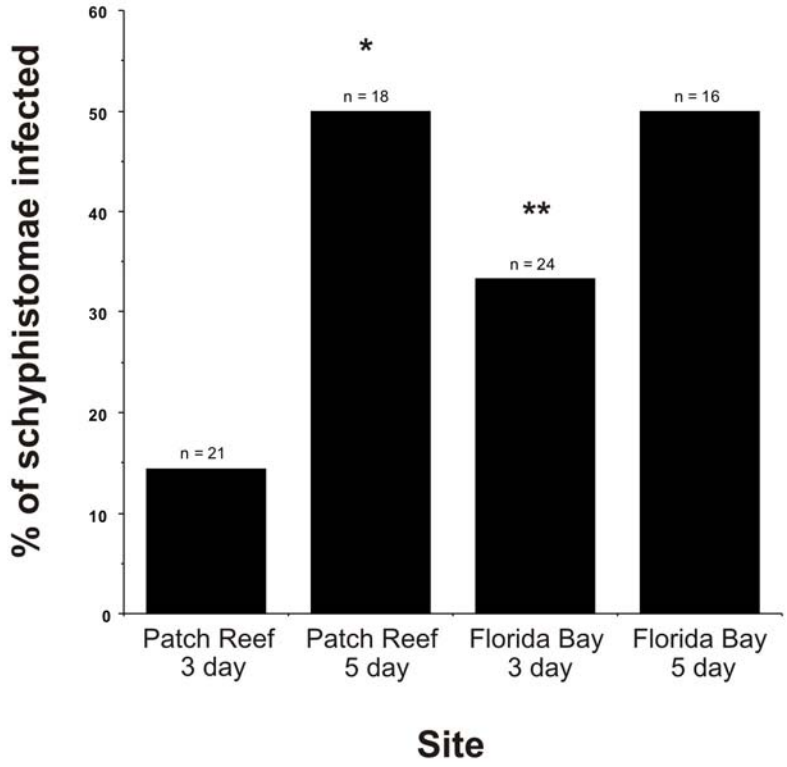


Figure 2.3 Percentage of scyphistomae of *Cassiopea xamachana* containing zooxanthellae following a 3 or 5-day exposure in Florida Bay or on a Admiral Patch Reef in the Atlantic Ocean off Key Largo in March 2003. The number of samples (number of polyps) is listed at the top of each histogram. * denotes a significant effect of exposure time when compared to the lowest exposure duration from that location. ** denotes a significant difference in infection rates for that time of exposure between locations.

CHAPTER 3

MULTI-YEAR, SEASONAL GENOTYPIC SURVEYS OF CORAL-DINOFLAGELLATE SYMBIOSES REVEAL PREVALENT STABILITY OR POST-BLEACHING REVERSION[2]

[2]Thornhill DJ, LaJeunesse TC, Kemp DW, Fitt WK, and GW Schmidt, *in press. Marine Biology*. Reprinted with permission of the publisher.

Introduction

Growing knowledge of the host-symbiont compositions of corals has caused conjecture about how they become modified in response to climate and/or environmental change (Buddemeier and Fautin 1993; Baker 2001; LaJeunesse 2005). The modern coral reef ecosystem has ancient origins, emerging in the early Cenozoic (Wood 1998). Reefs have persisted through dramatic shifts in temperature (Haq et al. 1987), yet extant corals are paradoxically sensitive to slight temperature increases of 1-2 ˚C above normal summer highs (Hoegh-Guldberg 1999; Hughes et al. 2003). Hypothetically, a change in the dinoflagellate symbiont to one better adapted to the prevailing environment could optimize the physiology of the holobiont association (Rowan and Powers 1991; Buddemeier and Fautin 1993). Annual seasonal monitoring of the resident symbiont populations in selected coral (Scleractinia) colonies could reveal how these symbiotic associations respond to seasonal and climatic environmental fluctuations.

Symbiodinium, the genus of dinoflagellate that associates with cnidarians, is genetically and physiologically diverse (Trench 1993, 1997; Rowan 1998; LaJeunesse 2001). The genus consists of eight genetically divergent sub-generic clades (A-H). Each clade comprises numerous "types" that are distinguishable genetically, ecologically, and biogeographically (reviewed by Baker 2003). The extent to which these are distinguished physiologically remains largely unknown. Among cultured *Symbiodinium* spp., broad differences in physiology have been observed (eg. Fitt 1984; Iglesias-Prieto and Trench 1994; Warner et al. 1999; Kinzie et al. 2001). Thermally tolerant *Symbiodinium* spp. are known from several clades (Tchernov et al. 2004). For instance, bleaching-resistant corals from the Great Barrier Reef harbor ITS type C15, whereas bleaching-susceptible coral colonies contained other clade C ITS types (LaJeunesse et al. 2003). Certain members of *Symbiodinium* clade D are described as stress tolerant because

they associate with corals from marginal habitats or those recovering from episodes of stress (Toller et al. 2001a,b; Chen et al. 2004). Some corals have slightly greater thermal tolerance when harboring clade D *Symbiodinium* (Baker et al. 2004; Rowan 2004), but this may decrease the growth rate of the host (Little et al. 2004).

The reproductive mode of the coral host affects how offspring become symbiotic. Most corals broadcast spawn aposymbiotic gametes into the water column (Szmant 1986), so symbionts must be acquired from the surrounding environment (horizontal acquisition) (van Oppen 2004). Each new generation creates the possibility for new host-symbiont combinations. In most corals where larvae are internally brooded symbionts are 'vertically' acquired, i.e. from the parent (van Oppen 2004). Specific combinations are maintained among various coral species and various *Symbiodinium* spp. The external environment and geographic location modulate these patterns of association (e.g. Loh et al. 2001; Rodriguez-Lanetty et al. 2001; LaJeunesse 2002, Iglesias-Prieto et al. 2004, LaJeunesse et al. 2004a,b).

While most host colonies possess a homogeneous *Symbiodinium* spp. population, some coral species may harbor multiple clade types within a single colony (Baker and Rowan 1997; Ulstrup and van Oppen 2003). Occasionally, mixed symbiont populations are found in colonies from intermediate or shallow reef habitats. The distribution of different symbionts within a single colony correlates with irradiance levels and may relate ultimately to differences in photophysiology among symbionts (Rowan et al. 1997). Few mixed symbiont populations have ever been observed in deep, low light environments (LaJeunesse 2002).

Even with evidence for physiological diversity among symbionts and change in some in hospite populations that reflects physical differences in the environment (e.g. Baker 2001), few long-term studies of symbiont population change have been reported (but see Goulet and

Coffroth 2003). To predict coral responses to future climate change information on how coral symbioses vary from season to season and year to year is needed. Change in the assemblage of symbiont types is one mechanism by which corals could acclimate to temporal changes in light, temperature and other abiotic factors (Buddemeier and Fautin 1993). To test this hypothesis, we examined the extent to which six species of reef-building corals from the western Atlantic (*Acropora palmata*, *A. cervicornis*, *Siderastrea siderea*, *Montastrea faveolata*, *M. annularis* and *M. franksi*) undergo changes in the relative abundances of different *Symbiodinium* types seasonally, over a five-year period. The six host species are principal contributors to reef accretion in the western Atlantic. All are broadcast spawners (Szmant 1986) that acquire zooxanthellae from the environment early in ontogeny and therefore are predicted to be open to infection by multiple types of *Symbiodinium* that might persist in varying amounts during colony maturation. An uncertain alternative is that mature corals could also remain capable of symbiont acquisition and can switch symbiont types accordingly.

If variance in abiotic factors, such as temperature and light, leads to variable symbioses, this pattern should be evident at the northern and southern limits of reef occurrence, where seasonality is most pronounced. This study examined symbioses in two northern reef systems, the Bahamas and Florida Keys, providing a comparison of symbiont responses to differing temperature extremes and anthropogenic impacts (more extreme in the Florida Keys than in the Bahamas) (Fitt et al. 2000; http://www.ndbc.noaa.gov/). Additionally, both regions experienced extensive loss of algal symbionts during the 1997-1998 mass coral-bleaching event, including the colonies examined in this study (Fitt et al. 2000). Within each region, sites were selected from different habitats and depths in order to track changes in symbiotic combinations reef-wide.

Methods

Coral tissue samples were collected by SCUBA quarterly (March, May, August, and November) from five species of scleractinian coral, *Acropora palmata* (Lamarck), *A. cervicornis* (Lamarck), *Montastrea faveolata* (Ellis and Solander), *M. annularis* (Ellis and Solander), and *M. franksi* (Gregory), from reefs off Lee Stocking Island, the Bahamas (24˚15'N; 76˚30'W) and the Upper Florida Keys (24˚59'N, 80˚22'W). Coral colonies were sampled once in 1998 and regularly between March 2000 and August 2004. An additional species, *Siderastrea siderea* (Ellis and Solander), was collected beginning in May 2002. The reefs sampled in the Bahamas included the shoreline Palmata Beach (1-3 m depth), inshore North Norman's Patch (2-4 m depth) and deep-water South Perry (12-15 m depth). Similarly, in the Florida Keys, the inshore Admiral patch reef (1-2 m depth), offshore Little Grecian Reef (3-4 m depth), as well as deep-water Conch (12 m depth) and Alligator (12 m depth) reefs were sampled. Certain species were not found in every environment sampled. For instance, *A. cervicornis*, *A. palmata*, and *S. siderea* did not commonly occur at any of the deep sites, whereas *M. franksi* was present only on deep reefs.

Six replicate colonies were identified and tagged for each available species to ensure that subsequent collection was from the same colony. *Acropora cervicornis* was the only species not collected in this manner; instead samples were obtained from six distinct colonies, but not necessarily the same colony during each sampling period. Approximately 5-cm diameter tissue samples were collected from 'boulder' corals via hammer and chisel, with care taken to ensure that the same relative position (i.e. the unshaded colony tops) was sampled each time. For 'branching' corals, approximately 10-cm lengths were snapped off by hand. Coral fragments

were placed in seawater-filled, pre-labeled plastic bags and transported immediately to the laboratory in an insulated cooler, where they were processed immediately.

Coral tissue was removed from 5 to 25 cm^2 of coral skeleton with a recirculating Water Pik^{tm} using filtered (0.45 μm) seawater. Tissue was completely removed with this method from the tops of *Montastrea* spp. and *Siderastrea siderea* fragments, the entire circumference of *Acropora cervicornis* at least 1 cm from the tip, and equal areas on the top and underside of *A. palmata* branches. This salt-water "blastate" was pulsed briefly (1-4 s) using a Brinkmann Instruments Polytron Kinematica Tissue Homogenizer^{TM} to disperse mucopolysacharides. *Symbiodinium* spp. cells were isolated from the remaining salt-water blastate via centrifugation in 50-ml tubes at 1000 *g* and the resulting algal pellet was preserved in DMSO Buffer (20% dimethyl sulfoxide and 0.25 M ethylene diaminetetraacetic acid (EDTA) in NaCl-saturated water) (Seutin et al. 1991). Samples from 1998 were lyophilized and stored at –20° C until extraction.

Nucleic acids were extracted using the Wizard® DNA preparation protocol (Promega) following the methods of LaJeunesse et al. (2003), with the following modifications. As an alternative to bead beating for cell lysis, samples from 1998 were briefly sonicated using a Heat Systems Ultrasonic Processor^{TM}. In some extractions, 0.3 M Tris, 2 mM EDTA, 0.7% SDS pH 7.5 was used in place of Nuclei Lysis Solution and 7.5 M ammonium acetate was used in place of Protein Precipitation Solution® (Promega).

The internal transcribed spacer 2 region (ITS 2) of nuclear ribosomal RNA was used to discriminate molecular types of *Symbiodinium* (LaJeunesse 2001, 2002). This region was amplified from the DNA extract for denaturing-gradient gel electrophoresis (DGGE) using primers "ITS 2 clamp" (5'CGCCCGCCGC GCCCCGCGCC CGTCCCGCCG CCCCCGCCC

GGGATCCATA TGCTTAAGTT CAGCGGT-3') and "ITSintfor 2" (5'GAATTGCAGA ACTCCGTG-3'). PCR amplification followed the "touchdown" thermal cycle protocol of LaJeunesse (2002). Products of these PCR reactions were checked by electrophoresis on agarose gels (1.0% agarose in 40 mM Tris-acetate, 1mM EDTA solution). Samples containing successfully amplified PCR products were subsequently electrophoresed on denaturing gradient gels (45-80% formamide, 8% polyacrylamide denaturing gradient gels; 100% consists of 7 *M* urea and 40% deionized formamide) and compared to reference samples of known ITS2 type following the protocol of LaJeunesse and Trench (2000), with the modifications of LaJeunesse et al. (2003).

The detection limits of DGGE analysis for the ITS 2 region in *Symbiodinium* spp. were tested using mixtures of different ITS types from cultured *Symbiodinium* spp. Cell counts (n=10 sample^{-1}) were performed using a hemacytometer and then equal numbers of fresh cultures of known ITS 2 type, alpha-numerically designated B1 and C2 (LaJeunesse 2001), were homogenized with a bead beater. After culture samples were mixed to give cell ratios of the two ITS types ranging from 1:152 (B1:C2) to 260:1 (B1:C2), DNA was extracted from the samples using the methodology described above. The ITS 2 region was amplified by PCR, and samples were analyzed on denaturing gels to determine the minimal population levels for which the two ITS types could be definitively identified.

Results

Patterns in Acropora spp. symbioses

The symbiotic patterns within coral colonies were tracked initially in samples from 1998 and seasonally between March 2000 and August 2004 for *Acropora palmata* and *A. cervicornis*.

Example diagnostic PCR-DGGE profiles of the *Symbiodinium* types detected in one colony of *A. palmata* and of *A. cervicornis* are shown (Fig.3.1). In both species of *Acropora*, symbiosis was always with *Symbiodinium* type A3, although mixed symbioses including type B1 were detected twice in this particular *A. cervicornis* patch (Fig. 3.1b). These examples illustrate stable associations between host and symbiont, the most common situation encountered in all host species.

The symbiont types detected in replicate colonies (n=6) of *Acropora palmata* and *A. cervicornis* per reef are compiled in pie charts from August 1998 to August 2004 (Fig. 3.2). Each pie chart represents one season and a 1/6 wedge of a pie chart designates each colony. Because PCR-DGGE is not a quantitative method, relative abundance above the minimum detection threshold (≥7%, see below) of a particular symbiont type cannot be precisely determined. As a result, detection of multiple types within a colony is illustrated by splitting a 1/6 wedge between the two types. Each row of pie charts was taken from the same replicate colonies unless otherwise noted. Data from 1998 were taken from the same reef, but not necessarily the same colonies.

At the detection level of DGGE (≥7%, see below), colonies of *A. palmata* were invariably symbiotic with *Symbiodinium* type A3 (Figs. 3.1a, 3.2a). This pattern occurred throughout the sampling in both the Bahamas (inshore patch reef, Palmata Beach, 1-3 m) and Florida Keys (offshore fore reef, Little Grecian Reef, 3 m). No change was observed in relation to seasonal fluctuations or following the 1997-98 bleaching event.

Colonies of *Acropora cervicornis*, while also stable in their symbioses with *Symbiodinium* type A3, were sometimes punctuated by the appearance of symbioses with mixed ITS types (types B1 or C3, Figs. 3.1b, 3.2b). In colonies where *Symbiodinium* types B1 or C3

were detected, type A3 was always also present; no sample was ever detected with a complete change in symbiont type. A mixed symbiosis was always short lived, as the interruptive symbiont type typically did not persist at detectable levels into the next sampling period. Most mixed symbiosis events were limited to a single colony, however during August 2001 all *A. cervicornis* colonies on North Norman's patch reef were detected harboring both type A3 and B1 *Symbiodinium*. Mixed symbioses events occurred in both regions investigated but did not appear to be correlated with seasonal fluctuations or to follow the 1997-98 bleaching event.

Patterns in Siderastrea siderea symbioses

Siderastrea siderea was added to this study in May 2002 to increase its scope beyond two host genera. Pie charts representing *S. siderea* symbiotic associations were compiled in the same method described for *Acropora* spp. symbioses, with each colony allocated a 1/6 wedge of the chart (Fig. 3.3). *Symbiodinium* types B5a, a specialist symbiont in the genus *Siderastrea* (LaJeunesse 2002), and C3 were detected as symbionts in *S. siderea* (Fig. 3.3). *Siderastrea siderea* associations were generally unchanged from May 2002 to August 2004 with only minor fluctuations of relative abundance. Colonies from the North Norman Patch Reef in the Bahamas showed no detectable change in symbioses with type C3. Similarly in the Florida Keys, colonies from the offshore Little Grecian Reef were unchanged in their symbioses with type B5a. Colonies from Admiral Patch Reef in Florida consistently harbored either type B5a (3 colonies) or type C3 (3 colonies), but occasionally, *S. siderea* colonies from Admiral Reef had mixed symbioses of both B5a and C3 (Fig. 3.3). In all such cases, the colony returned to the previously established symbiont type within one to two sampling periods and no permanent change of symbiont type was ever observed.

Patterns in Montastrea spp. symbioses

Symbiodinium spp. associations with species of *Montastrea faveolata*, *M. annularis*, and *M. franksi* were monitored initially in Florida during August 1998 and in six tagged from the Bahamas and Florida Keys colonies per reef seasonally between March 2000 and August 2004. These data were compiled as pie charts as described for *Acropora* spp., with each colony allocated a 1/6 wedge of a chart and each chart representing one time period (Figs. 3.4, 3.5).

Symbioses in *Montastrea faveolata* remained stable at virtually all sites in the Bahamas and the Florida Keys (Fig. 3.4). Individual colonies usually showed fidelity over time to one particular *Symbiodinium* partner and changing symbiont types was rare.

Colonies of *Montastrea faveolata* from the Bahamas contained either type B1 or C12 *Symbiodinium*. Shallow colonies from North Norman's Patch Reef (4 m) were symbiotic with type B1 throughout the five years of monitoring except for two colonies with a mix of B1 and C12 from late 2002 to early 2003. *Montastrea faveolata* from deep South Perry Reef (12 m) in the Bahamas were mixed in their complement of *Symbiodinium* clades. Two colonies harbored type B1, which predominantly inhabits shallow colonies of the same coral species throughout the Caribbean (LaJeunesse 2002), or a mix of B1 and C12. The remaining four South Perry colonies always contained only the typical deep-water *Symbiodinium* type C12 (Figure 3.4).

In the Florida Keys, *Montastrea faveolata* had stable symbioses with *Symbiodinium* type B1 throughout the study at all habitats and depths with several exceptions (Fig. 3.4). Following the bleaching event of 1997-98, shallow fore reef colonies (3 m) were detected with types B10 and D1a in addition to B1. Additionally, several tagged colonies of *M. faveolata* briefly harbored mixed communities with D1a (Little Grecian reef, 3 m) or C3 (Conch Reef, 12 m)

during 2000 but otherwise exhibited stable associations with clade B *Symbiodinium* (Fig. 3.4). Finally, on Conch Reef, one colony of *M. faveolata* harbored type B10 during all years it was sampled, while type B1 was detected in all of the other colonies (n=5) sampled (Fig. 3.4).

Montastrea annularis from the Bahamas, as with the majority of hosts sampled, had symbioses dominated by a single symbiont type, in this case type B1 (Fig. 3.5a). Shallow colonies from North Norman's Patch reef (4 m) were invariable while deep colonies from South Perry reef (12 m) had several brief instances of mixed B1 and C12 symbioses.

Shallow colonies of *Montastrea annularis* from the Florida Keys (inshore Admiral Patch reef at 1-2 m and offshore Little Grecian fore reef at 3 m) experienced temporal changes in the symbiont partner that did not occur in other species sampled at the same sites (Fig. 3.5a). Mixtures of B1 and D1a were observed in preliminary samples of *M. annularis,* taken in August 1998 on the fore reef at 3 m (Fig. 3.5a). Subsequent sampling from tagged colonies revealed temporal changes in the identity and proportion of symbiont types D1a, C3, B1 (Admiral Reef) or D1a, B1, and B10 (Little Grecian) from March 2000 until August 2002, when a stable dominance of type B1 (Admiral Reef) or B10 (Little Grecian Reef) was reached, four to five years after the 1997-8 El Niño episode ended (Fig. 3.5a). A PCR-DGGE profile taken from one tagged colony on Little Grecian reef is shown to further illustrate the shift from D1a to B10 (Fig. 3.6). This shift from symbioses dominated by type D1a to clade B *Symbiodinium* was not observed in deep Florida colonies (Conch Reef at 12 m) which were invariably associated with type B1.

Montastrea annularis is known to harbor heterogeneous *Symbiodinium* spp. populations in different regions of a colony (e.g. Rowan et al. 1997). As a result, care was taken to ensure that the same relative colony position was sampled each season. Additionally, while type D1a

Symbiodinium was common in the "tops" of certain *M. annularis* colonies in 1998 and between 2000 and 2002 (Fig. 3.5a), this lineage was never observed during intensive sampling of the same colony's tops and sides from August 2002 to August 2005 (Appendix A).

Colonies of *Montastrea franksi* only occurred in significant numbers on deep reefs in the Bahamas and Florida Keys. On South Perry reef in the Bahamas (12 m), symbioses were with types B1 and C12, the same types found in other *Montastrea* species in this region (Fig. 3.5b). Four colonies were found to have symbiosis with C12 and the remaining two had B1, although mixed symbioses with C12 and B1 were common in many colonies. During March and May 2003 all colonies harbored both type B1 and C12 *Symbiodinium*.

Colonies of *Montastea franksi* on the deep fore reefs in Florida (Conch and Alligator reefs) possessed either D1a, B1, B10, C3 and/or combinations of these during 2000 and 2001 before returning to B1 (on Conch Reef, 1 colony on Alligator reef) or C3 (five colonies on Alligator Reef) by the end of the study (Fig. 3.5b). Similar to the shifts in *M. annularis*, most transitions in symbiont identity ended in 2002, three to five years after the 1997-8 El Niño event (Fig. 3.5b).

Detection limits of DGGE

The sensitivity limits of PCR-DGGE were established for identifying mixed populations of *Symbiodinium* spp. types within host tissues. The sensitivity of denaturing gradient gel electrophoresis for the ITS2 region from various ratios of cultured *Symbiodinium* types B1 and C2 is shown (Fig. 3.7). Type C2 was detected when present at ≥3.8% of the total sample whereas type B1 was detected less effectively at a proportion of ≥6.3%. Therefore, we conclude

that types occurring in proportions below 5 to 10 % of the total symbiont population will not be detected by this protocol.

Discussion

Most coral colonies investigated in this study experienced little variation in their dominant symbiont ITS2 type over the course of this six-year study. Hence, stable associations between hosts and particular symbionts seem to be common (see also Goulet and Coffroth 2003). This stable pattern persisted in most colonies following the 1997-1998 bleaching event, which negatively impacted these colonies, and through the acute seasonal fluctuations experienced in the Bahamas and Florida Keys. Coral colonies with such consistent symbioses appear unlikely to respond to environmental shifts by changing to alternate lineages of *Symbiodinium* spp. This result contrasts with the pattern reported by Baker et al. (2004), where some species of corals putatively shifted to thermal-tolerant clade D symbiont types following episodes of severe bleaching. While certain *Montastrea* spp. colonies in this study experienced change to type D1a following bleaching, most colonies did not. The discrepancy between the data presented by Baker et al. (2004) and this study may be explained by the different methodologies employed. Because Baker et al. (2004) did not repetitively sample tagged colonies, the shift to symbiosis with clade D types may be a result of differential mortality of coral hosts harboring clade C vs. clade D types. Alternatively, the severity of the 1997-98 bleaching event may have been insufficient to induce a change in symbiosis for the majority of the colonies in this study.

The stable symbioses observed in *Acropora palmata*, *A. cervicornis*, *Siderastrea siderea*, and many *Montastrea* spp. colonies might be explained by one or a combination of several possibilities: 1) competitive exclusion; in which differential growth rates between types would

lead to the ultimate displacement of slower growing species or strains (e.g. Fitt 1985); 2) molecular compatibility and/or recognition in new symbiont recruitment (host-specificity/selection) (e.g. Gates et al. 1995, Reynolds et al. 2000; Rodriguez-Lanetty et al. 2004) may restrict the variation of host-symbiont combinations; the five types of *Symbiodinium* associated with *Montastraea* spp. represent a small sub-set of the total diversity of *Symbiodinium* in these ecosystems (see also LaJeunesse 2002); 3) preferential expulsion or demise of symbionts that are directly perturbed physiologically under environmental stress; 4) differential survival of symbionts in response to altered metabolism of host cells; 5) chemical inhibition: established symbiont types may exclude competitors through the release of chemical inhibitors; 6) incumbent advantage: difficulties faced by the symbiont during the initial stages of infection may prevent successful colonization of host tissues that are already populated. For example, initial symbiont populations in laboratory-controlled infections declined drastically before subsequent growth (Schoenberg and Trench 1980; Colley and Trench 1983). This infection "hurdle" may make the successful introduction of new types rare, or impossible. Our inability to distinguish the relative importance of these possibilities, or unrealized alternatives, in governing specificity highlights our general lack of knowledge about this system.

All three *Montastrea* spp. in Florida experienced changes in their symbiont composition. Most notable was the disappearance of symbiont D1a during our survey. This phenomenon appears to be a reversion to pre-1997-98 symbioses, a re-sorting process that took place over a number of years before stabilizing with symbionts most commonly encountered at these and other locations around the Caribbean. Most significantly, the slow change from dynamic multi-cladal symbiont assemblages to a single dominant symbiont type occurred in the absence of any stressor (see also Ware et al. 1996). The slow change is consistent with previous studies. Toller

et al. (2001b) found no significant return to the original symbiont after nine months in experimentally stressed corals, where D-type *Symbiodinium* had become established. The change observed in *Montastrea* spp. symbioses is consistent with succession following disturbance, a phenomenon well documented in the ecological literature (Connell and Slatyer 1977).

It is important to recognize that the three species of *Montastrea* in this study are known to harbor multiple types of *Symbiodinium* within a single colony (Rowan et al 1997; Toller et al. 2001a). The flexible symbiotic associations in these species appears to represent one end of a continuum between changeable, multi-partner associations and stable, highly specific associations (Baker 2003).

Displacement of one type of symbiont by another in a host has been shown experimentally to involve overgrowth by the competitive dominant (Fitt 1985) and/or disappearance of the less resilient type in stressful conditions (Toller et al. 2001b). Berkelmans and van Oppen (2004) found that a type of clade D displaced a clade C in corals transplanted from a clear water habitat in the southern Great Barrier Reef (GBR) to a more turbid and warmer inshore reef in the central section of the GBR, after these had bleached. Any stress leading to the loss of the dominant *Symbiodinium* type may allow certain clade D *Symbiodinium* to flourish (Baker 2001; Toller 2001a; present study). While more information on the ecology and physiology of clade D types is required, their occurrence in hosts recovering from bleaching and/or in colonies from high temperature or high turbidity habitats (Toller et al. 2001a,b; van Oppen et al. 2001; Chen et al. 2003; Baker 2004) suggests that some are opportunistic, stress-tolerant, host-generalists. Populations of *Symbiodinium* clade D may persist at low

concentrations within host tissues (Ulstrup and van Oppen 2003) and/or possibly in the external environment (e.g. in gastro-vascular cavities, on the host surface, water column, benthos, etc.).

The importance of the symbiotic association as a primary energy source to corals is well established (Odum and Odum 1955; Muscatine and Porter 1977; Muscatine 1990). Change in *Symbiodinium* types following bleaching (in this case to type D1a) may impart the temporary benefit of thermal tolerance (Rowan 2004) but may impair growth rates and reproduction of hosts until pre-bleaching symbiont types are re-acquired (Szmant and Gassman 1990; Little et al. 2004). Since greater frequency and intensity of ENSO events are predicted (Dunbar et al. 1994), a long symbiont reset time might preclude complete recovery (Wellington et al. 2001). Comparing the long-term viability of various host and symbiont combinations under different environmental conditions will be a crucial step towards testing the 'adaptive bleaching hypothesis' (Fautin and Buddemeier 1993) and understanding the chronic processes leading to the demise of reef corals worldwide.

Major changes in symbiont dominance with time were only observed in *Montastrea annularis* and *M. franksi* at certain reefs in the Florida Keys. Greater environmental variability in this higher latitude reef system may explain higher within-colony diversity and temporal instability compared to the colonies from the Bahamas. Floridian reefs are at the northern latitudinal boundary for many Caribbean coral species (Porter and Tougas 2001), and seasonal temperature extremes in the Florida Keys are greater than those in the Bahamas (Fitt et al. 2000; http://www.ndbc.noaa.gov/). Additionally, negative terrestrial influences (freshwater runoff with high nutrients, sediments, pesticides, pathogens, plus other anthropogenic stressors) are chronic impacts to reefs in the Florida Keys (LaPointe and Clarke 1992; Porter and Porter 2002).

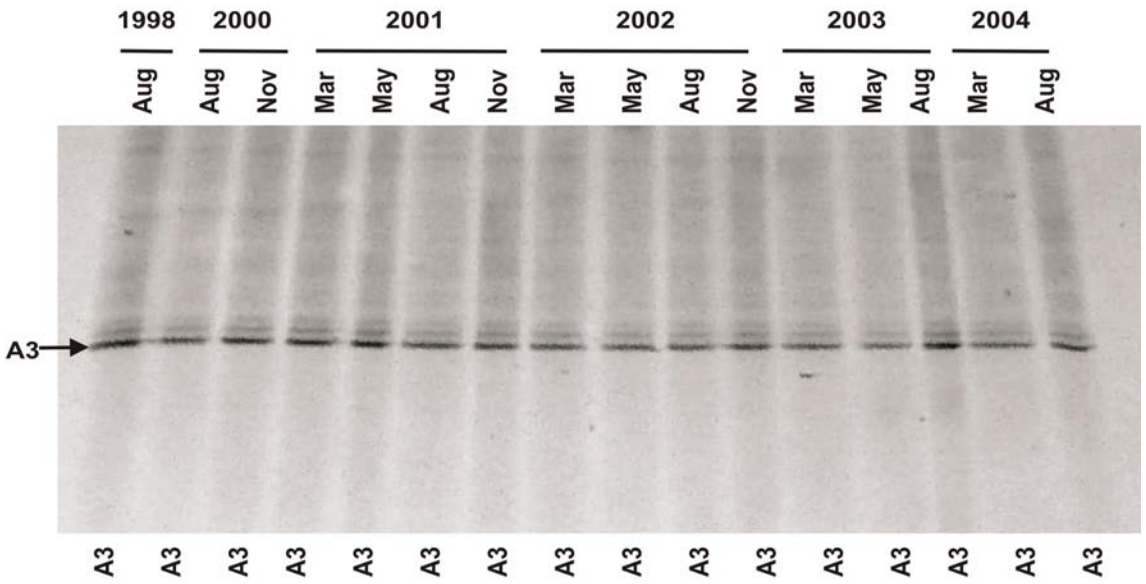
Symbiont associations in *Montastrea annularis* in Florida varied more in shallow than in deep-water habitats, perhaps due to less environmental variation on deeper reefs (see also LaJeunesse 2002). Visible light and UV radiation are attenuated with depth and variance in mean daily temperature is less extreme in the deep reefs (e.g. Fitt et al. 2000). The apparent lower symbiont diversity in deep environments (LaJeunesse 2002) may further limit the occurrence of mixed symbiont populations. A similar hypothesis was invoked to explain the prevalence of *Symbiodinium* Clade D in colonies of *M. annularis* in near-shore Caribbean habitats compared to colonies living on offshore reefs (Toller et al. 2001a,b).

The DGGE sensitivity limits of approximately 7% of the total *Symbiodinium* spp. population still enables an accurate characterization of the symbiont types that provide the primary physiological contribution to the holobiont. This method can also generally detect symbiont types that might have supplementary and/or environmental acclimatization functions. However, it is likely that additional symbiont ITS types co-exist in many colonies below this ~7% sensitivity threshold and go undetected. There also may be a slight bias in the DGGE methodology for the detection of certain *Symbiodinium* clades, which could be a result of differences in ribosomal gene repeat number and/or disproportionate primer annealing during PCR amplification. Despite an observed slight bias for C types over B types in reconstruction experiments, all classes of *Symbiodinium* are detected when their DNA forms at least 5 to 10 % of the sample. Nonetheless, when change occurs in a colony's dominant symbiont type it currently is impossible to determine whether the new symbiont originates from 'shifting' to an exogenous type acquired from the outside environment or preferential proliferation of a pre-existing endogenous type.

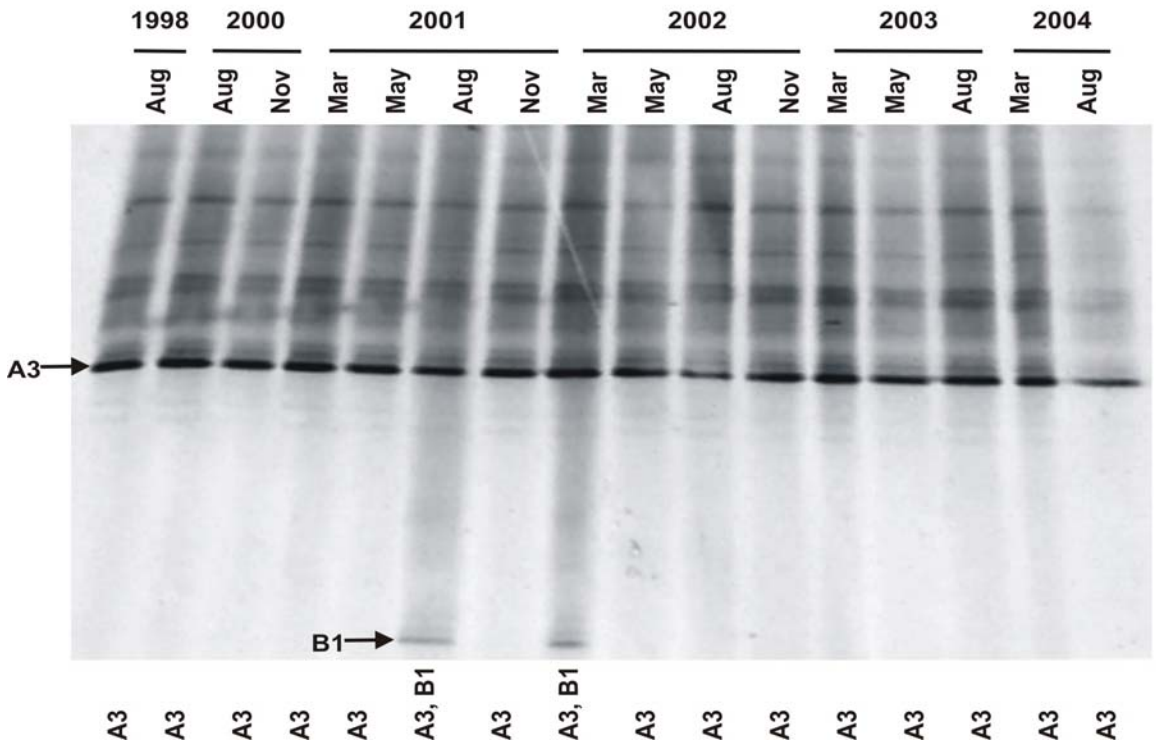
Recent episodes of mass bleaching and mortality have led to dire predictions about the future persistence of reefs (Hoegh-Guldburg 1999; Hughes et al. 2003). While reef corals may acclimate to increases in global temperature by experiencing changes in their dominant symbiont type, the results of this study suggest that such phenomena are not a widespread response to coral bleaching. Although increasingly severe future bleaching events may result in changes to more thermally adapted symbionts, we predict that thermal resistance is as likely to be acquired via acclimatization or adaptation on the part of either the coral host or the dinoflagellate symbiont in stable symbiotic associations.

Figure 3.1 (A) PCR-DGGE profile of the *Symbiodinium* ITS 2 region collected from a single colony of *A. palmata* from Palmata Beach reef (depth 3 m) in the Bahamas. Gel profile tracks this colony's symbiosis from August 1998 to August 2004 and is presented as a reverse image. Diagnostic band labeled for type A3. (B) PCR-DGGE profile of the *Symbiodinium* ITS 2 region of symbionts from the same stand of *A. cervicornis* from North Norman's Patch reef (depth 4 m) in the Bahamas. Gel profile tracks this stand's symbiosis from August 1998 to August 2004 and is presented as a reverse image. Diagnostic bands labeled for type A3 and type B1. Faint, repeated bands in each profile are possibly rare intragenomic variants and heteroduplexes created between them and dominant A3 sequence.

(A) *Acropora palmata*



(B) *Acropora cervicornis*



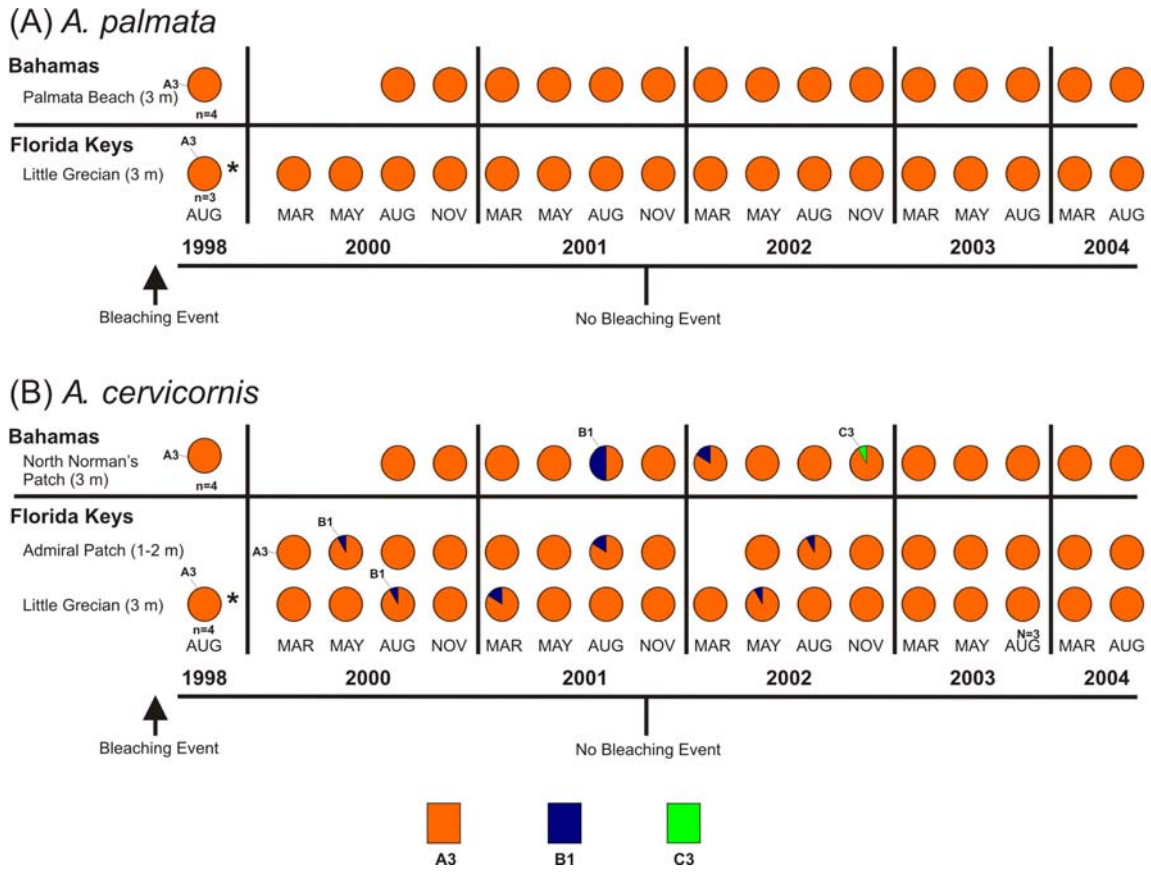


Figure 3.2 *Acropora palmata* and *A. cervicornis* ITS 2 *Symbiodinium* types detected in colonies from the Bahamas and Florida Keys from August 1998 to August 2004. Colonies (n=6) listed by region, reef type, and depth as rows of pie charts. Pie charts for samples from non-replicate colonies marked with an asterisk. Occasionally, fewer than 6 replicates were available. These instances are demarcated by 'n= the number of replicates' below a relevant chart. Appearance of a new symbiont type in each set of colonies is designated with its alpha-numeric clade designation. (A) *A. palmata*. (B) *A. cervicornis*

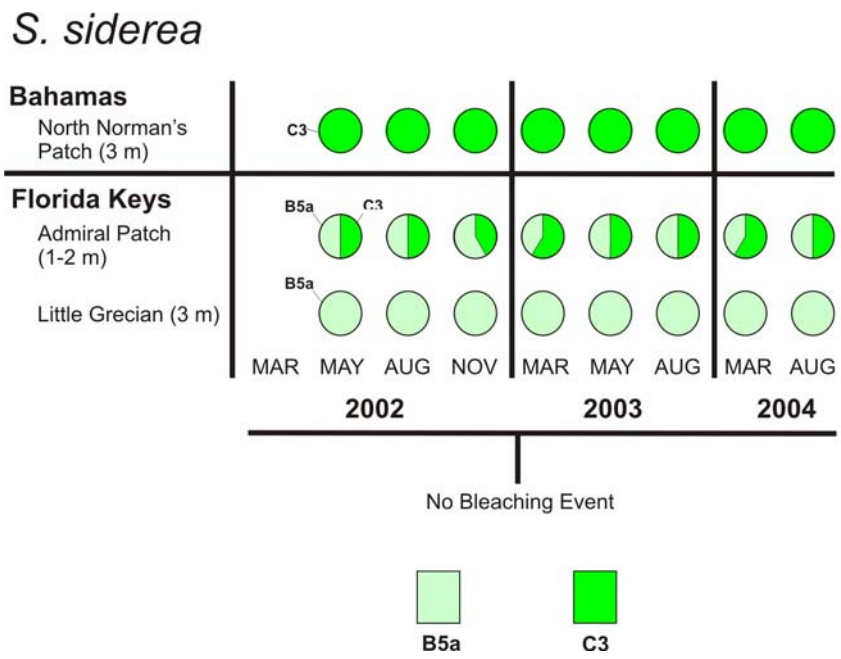


Figure 3.3 *Siderastrea siderea* ITS 2 *Symbiodinium* types detected in colonies from the Bahamas and Florida Keys from May 2002 to August 2004. Colonies (n=6) are listed by region, reef type and depth as rows of pie charts. Appearance of a new symbiont type in each set of colonies is designated with its alphanumeric name

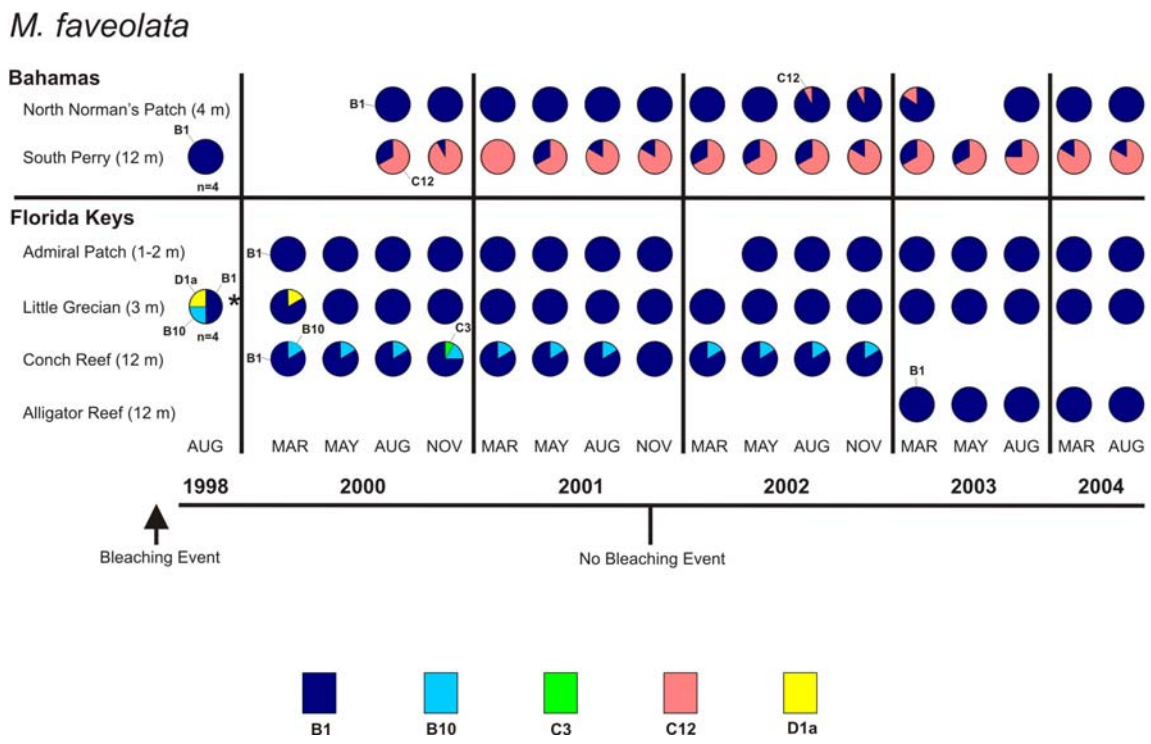


Figure 3.4 *Montastrea faveolata* ITS 2 *Symbiodinium* types detected in colonies from the Bahamas and Florida Keys from August 1998 to August 2004. Colonies (n=6) are listed by region, reef type, and depth as rows of pie charts. Occasionally, fewer than 6 replicates were available. These instances are demarcated by 'n= the number of replicates' below a relevant chart. Appearance of a new symbiont type in each set of colonies is designated with its alpha-numeric name

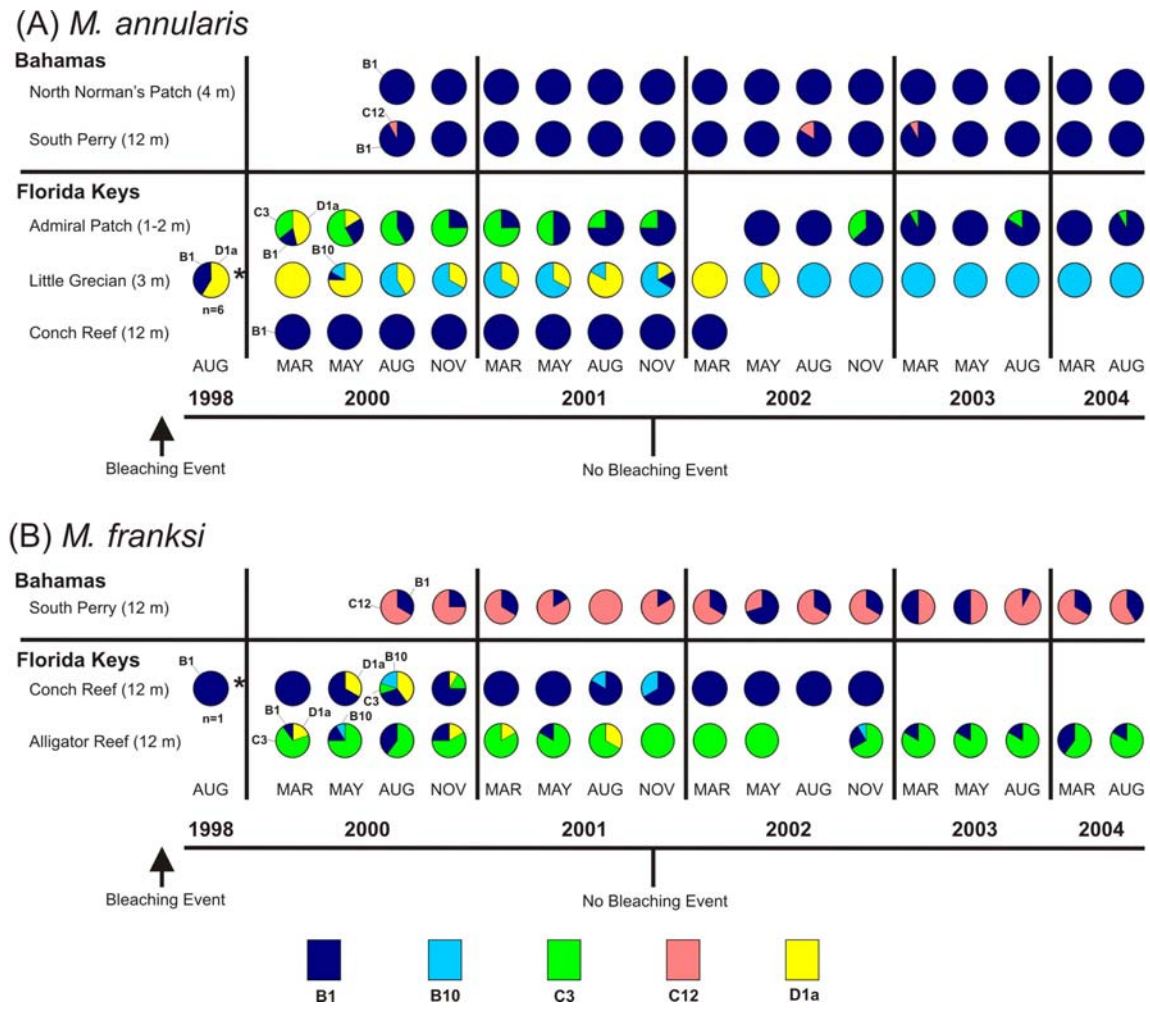


Figure 3.5 *Montastrea annularis* and *M. franksi* ITS 2 *Symbiodinium* types detected in colonies from coral reefs of the Bahamas and Florida Keys from August 1998 to August 2004. Colonies (n=6) listed by region, reef type and depth as rows of pie charts. Occasionally, fewer than 6 replicates were available. These instances are demarcated by 'n= the number of replicates' below a relevant chart. Appearance of a new symbiont type in each set of colonies is designated with its alpha-numeric name. (A) *M. annularis*. (B) *M. franksi*

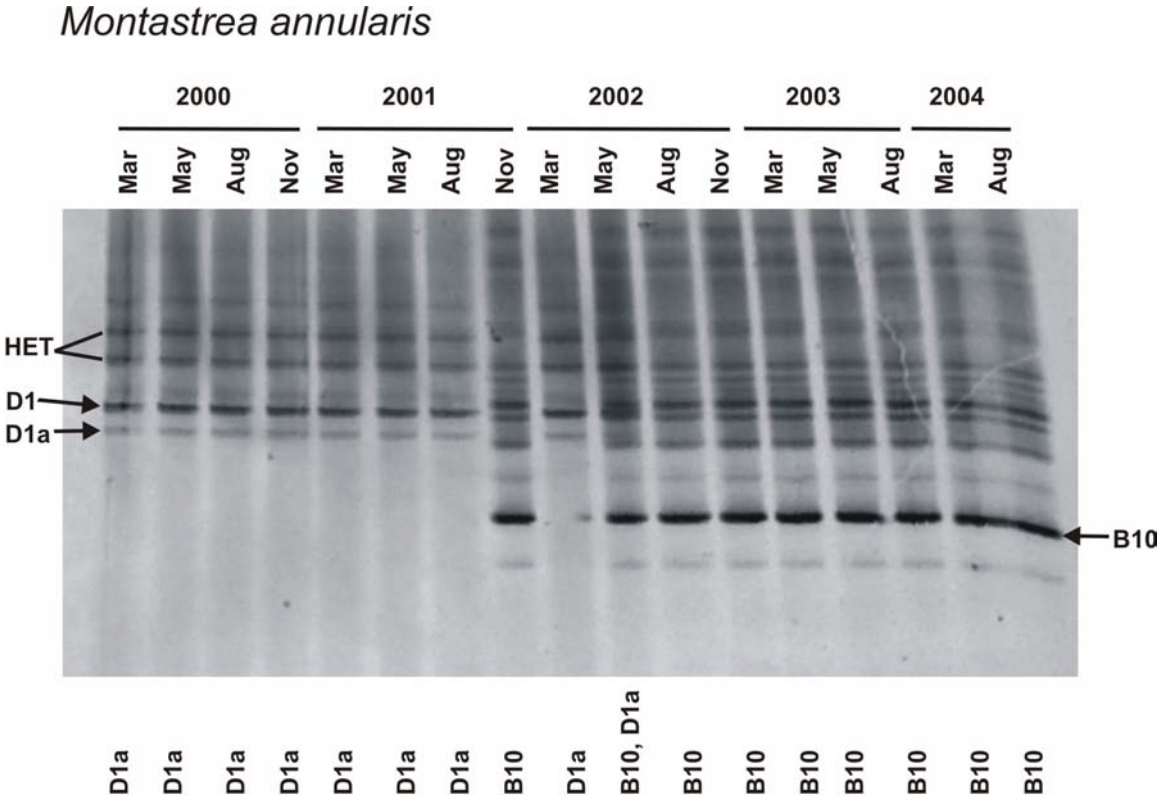


Figure 3.6 PCR-DGGE profile of the *Symbiodinium* ITS 2 region from colony 3 of *Montastrea annularis* from Little Grecian fore reef (depth 3 m) in the Florida Keys. Gel profile tracks this colony's symbiosis from August 1998 to August 2004. Profiles presented as a reverse image. Diagnostic bands labeled for type D1a and type B10. Faint repeatable bands above the prominent B10 band are likely a combination of rarer intragenomic variants and heteroduplexes. Faint band below the B10 band represents vestiges of unconverted ancestral B1 sequence repeats. The "D1a" fingerprint contains two codominant sequence variants D1 and D1a. The D1a band distinguishes this symbiont from another clade D symbiont that contains only the D1 sequence (thus has only one prominent band in its fingerprint profile)

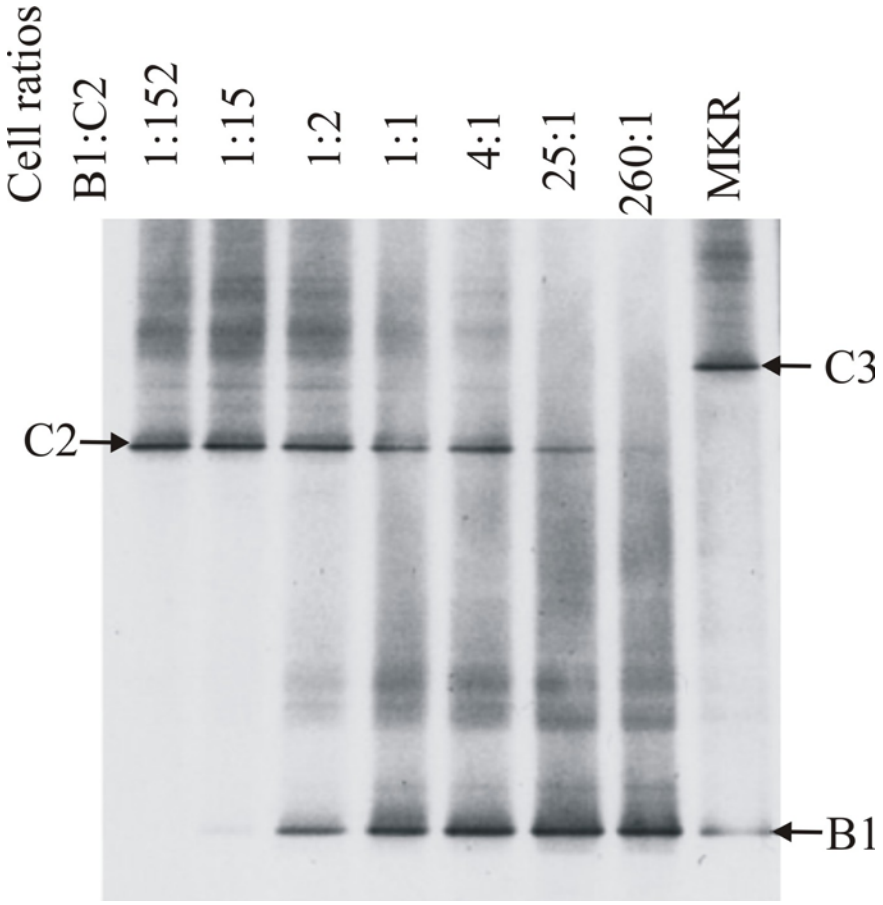


Figure 3.7 *Symbiodinium* spp. PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis) profile of ITS 2 (internal transcribed spacer) region from various ratios of representatives from two cultured clades, *Symbiodinium* spp. B1 and C2 (LaJeunesse 2001). Gel profile presented as a reverse image. An ITS type was detectable if it represented at least ~7% of the total sample (6.3% for B1; 3.8% for C2). Ratios were determined by cell counts (n=10) of homogenized cultures. Standards (MKR) in lane 8 are pooled PCR amplifications from cultured B1 and C3 types

CHAPTER 4

INTRACOLONIAL *SYMBIODINIUM* SPP. DISTRIBUTION PATTERNS IN *MONTASTREA ANNULARIS* FROM FLORIDA[3]

[3] Thornhill DJ and WK Fitt. To be submitted to *Bulletin of Marine Sciences*.

Introduction:

The *Montastrea annularis* species complex (Lopez et al. 1999) has an unusual pattern of symbiotic association; different *Symbiodinium* clades are harbored in different regions of a coral colony (Rowan and Knowlton 1995, Rowan et al. 1997). Hypothetically, this pattern is a result of physiological tolerances for light among *Symbiodinium* clades (Rowan et al. 1997). Putatively light tolerant clades A and B are typically found on *M. annularis* colony tops whereas light intolerant clade C occurs in the colony sides (Rowan et al. 1997). Furthermore, rotation of a coral colony resulted in corresponding shifts in the endosymbiont community (Rowan et al. 1997). Few other host species are known to have a predictable pattern of intracolonial variation in symbiont population (see also Ultsrup and van Oppen 2003).

Seasonal sampling of the *Symbiodinium* spp. population from scleractinian coral colonies revealed greater variability in the symbiont population of *Montastrea annularis* than in other host species (Thornhill et al. 2005). Thornhill et al. (2005) showed seasonal variability in the presence of clade C on *M. annularis* colony tops from Florida. Clade C was detected in August and/or November in 2002-2004 but absent the remainder of the year. Based on this observation, we hypothesized that seasonal shifts occur in the *Symbiodinium* spp. community. To test this idea, we identified *Symbiodinium* ITS2 types from the tops and sides of *M. annularis* colonies of Admiral Patch Reef in Key Largo Florida from May 2003 to September 2005. We also measured the Symbiodinium type in the tops and sides of *M. faveolata f*rom adjacent colonies on Admiral Reef and from *M. annularis* and *M. faveolata* colonies from the nearby reef Little Grecian Fore-Reef to observe whether similar intracolonial patterns occur throughout this region. Finally, we repeated the colony rotation experiments of Rowan et al. (1997).

Methods:

Coral tissue samples were collected by SCUBA from the bolder star coral *Montastrea annularis* (Ellis and Solander) on the inshore Admiral patch reef (1-2 m depth) off the Upper Florida Keys (24˚59'N, 80˚22'W). Coral colonies were sampled regularly between May 2003 and September 2005. In March 2004, Additional samples were taken from *M. annularis* and *M. faveolata* (Ellis and Solander) on Admiral patch reef and the offshore Little Grecian Reef (3-4 m depth). Approximately 5-cm diameter tissue samples were collected from the unshaded colony tops and the shaded colony sides off each colony with care taken to ensure that the same relative position was sampled each time. Coral fragments were placed in seawater-filled, pre-labeled plastic bags and transported immediately to the laboratory in an insulated cooler, where they were processed immediately.

Three colonies of *M. annularis* were selected for colony rotation in May 2004. Four 20-cm diameter pieces of each colony, consisting of a light exposed top and a well-shaded colony side, were removed. One of these fragments from each colony was returned immediately for symbiont type identification. The remaining three pieces per colony were rotated 90° and fixed in place with an epoxy. One rotated fragment was removed from each colony in September 2004, March 2005 and September 2005 to determine how the symbiont type had changed for each colony. Coral fragments were placed in seawater-filled, pre-labeled plastic bags and transported immediately to the laboratory in an insulated cooler, where they were processed immediately.

Coral tissue was removed from 5 to 25 cm^2 of coral skeleton with a recirculating Water Pik^{tm} using filtered (0.45 μm) seawater. This salt-water "blastate" was pulsed briefly (1-4 s)

using a Brinkmann Instruments Polytron Kinematica Tissue Homogenizer™ to disperse mucopolysacharides. *Symbiodinium* spp. cells were isolated from the remaining salt-water blastate via centrifugation in 50-ml tubes at 1000 *g* and the resulting algal pellet was preserved in DMSO Buffer (20% dimethyl sulfoxide and 0.25 M ethylene diaminetetraacetic acid (EDTA) in NaCl-saturated water) (Seutin et al. 1991). Samples from 1998 were lyophilized and stored at – 20° C until extraction.

Nucleic acids were extracted using the Wizard® DNA preparation protocol (Promega) following the methods of LaJeunesse et al. (2003), with the following modifications. As an alternative to bead beating for cell lysis, samples from 1998 were briefly sonicated using a Heat Systems Ultrasonic Processor™. In some extractions, 0.3 M Tris, 2 mM EDTA, 0.7% SDS pH 7.5 was used in place of Nuclei Lysis Solution and 7.5 M ammonium acetate was used in place of Protein Precipitation Solution® (Promega).

The internal transcribed spacer 2 region (ITS 2) of nuclear ribosomal RNA was used to discriminate molecular types of *Symbiodinium* (LaJeunesse 2001, 2002). This region was amplified from the DNA extract for denaturing-gradient gel electrophoresis (DGGE) using primers "ITS 2 clamp" (5'CGCCCGCCGC GCCCCGCGCC CGTCCCGCCG CCCCCGCCC GGGATCCATA TGCTTAAGTT CAGCGGT-3') and "ITSintfor 2" (5'GAATTGCAGA ACTCCGTG-3'). PCR amplification followed the "touchdown" thermal cycle protocol of LaJeunesse (2002). Products of these PCR reactions were checked by electrophoresis on agarose gels (1.0% agarose in 40 mM Tris-acetate, 1mM EDTA solution). Samples containing successfully amplified PCR products were subsequently electrophoresed on denaturing gradient gels (45-80% formamide, 8% polyacrylamide denaturing gradient gels; 100% consists of 7 *M* urea and 40% deionized formamide) and compared to reference samples of known ITS2 type

following the protocol of LaJeunesse and Trench (2000), with the modifications of LaJeunesse et al. (2003).

Results and Discussion:

Seasonal patterns of intracolonial variation in *Montastrea annularis*

Symbiotic associations in the tops and sides of *M. annularis* colonies from May 2003 to September 2005 are shown as compiled pie charts (Fig. 4.1). Each pie chart represents one season and a 1/6 wedge of a pie chart designates each colony. Because PCR-DGGE is not a quantitative method, relative abundance above the minimum detection threshold (≥7%; Thornhill et al. 2005) of a particular symbiont type cannot be precisely determined. As a result, detection of multiple types within a colony is illustrated by splitting a 1/6 wedge between the two types (or 1/10 – 1/12 wedge depending on the number of samples available, see Fig. 4.1).

Contrary to our hypothesis, colonies do not appear to have seasonal variation in symbiotic associations with clade C *Symbiodinium*. Instead, *Symbiodinium* spp. types appear to be generally distributed according to the pattern described by Rowan and Knowlton (1995) with ITS type B1 on the colony tops and ITS type C3 on the colony sides. However, Clade A *Symbiodinium* was not observed in any *Montastrea* spp. colonies, suggesting that there may be regional differences in symbiotic associations (see LaJeunesse et al. 2004b). While limited deviation from this general distribution pattern occurs, it does not appear to be a result of seasonal changes in temperature and light intensity. Instead variation in the zonation of symbiont types appears to be more random and is perhaps a result of sampling error or microhabitat differences within a coral colony.

Symbiotic associations in the tops and sides of *Montastrea* spp. colonies from shallow Florida reefs

The symbiotic associations in the tops and sides of *M. annularis* from Little Grecian Reef were measured in March 2004 (Figure 4.2a). These colonies exclusively harbored ITS type B10, a specialist *Symbiodinium* type found only in *Montastrea* spp. corals. No variation in symbiont type was detected between the tops and sides of these colonies.

The *Symbiodinium* types found in the tops and sides of colonies of *M. faveolata* were measured on Admiral Patch Reef and Little Grecian Fore Reef in March 2004 (Fig. 4.2b,c). On Admiral Reef, the tops of all *M. faveolata* colonies harbored only type B1. However, of four out of same six *M. faveolata* colonies harbored mixed populations of *Symbiodinium* types B1 and C3 on the colony sides (Fig. 4.2b). The remaining two colonies harbored only type B1. Colonies of *M. faveolata* from Little Grecian Reef harbored only ITS type B1 regardless of the colony position sampled (Fig. 4.2c).

Differences in the top - side symbioses of *M. annularis* and *M. faveolata* were encountered in both species at the inshore Admiral Patch Reef, but in neither species at the offshore Little Grecian Reef. One likely explanation for this phenomenon is the difference in water clarity and degree of shading between these two reefs. Admiral Reef is an inshore reef (~3 miles offshore within the turbid Hawk Channel) which regularly has poor visibility. Additionally, colonies are surrounded by various species of gorgonians that provide additional shading. Little Grecian Reef (~5 miles offshore) typically has much better water clarity and colonies are not surrounded by gorgonian spp. The regularly lower light intensities of Admiral Reef may select for the putatively light intolerant clade C *Symbiodinium* (Rowan et al. 1997) resulting in mixed symbiont populations.

Colony rotation experiment

Prior to colony rotation, distribution of *Symbiodinium* types followed a pattern similar to that described by Rowan and Knowlton (1995). Colony tops contained type B1 and colony sides contained type C3 (2 colonies) or a mix of type C3 and type B1 (1 colony) (Fig. 4.3). In September 2004, four months after rotation, rotated colonies all showed considerable visible bleaching on the new colony tops (former colony sides) (Fig. 4.4). Surprisingly, colonies were detected as having a uniform population of ITS type C3 *Symbiodinium*; type B1 was not detected in any of the rotated colonies (Fig. 4.3). By March 2005, all rotated colonies had recovered to their original brown color. Symbiont populations were either C3 (2 colonies) or B1 (l colony) on the colony tops; similarly, symbiont populations were either C3 (2 colonies) or B1 (l colony) on the colony sides (Fig. 4.3). However presence of B1 was on the top of colony #2 and the side colony #1 so the pattern of symbiont distribution was not uniform between colonies. Colonies sampled in September 2005 had regained the distribution of *Symbiodinium* types observed in the unrotated colonies, with a dominance of B1 on the colony tops and C3 on the colony sides (Fig. 4.4). This result further confirms the pattern described by Rowan et al. (1997). It appears that type B1 is a superior competitor (see Fitt 1984) to type C3 in the light exposed tops of shallow *M. annularis*.

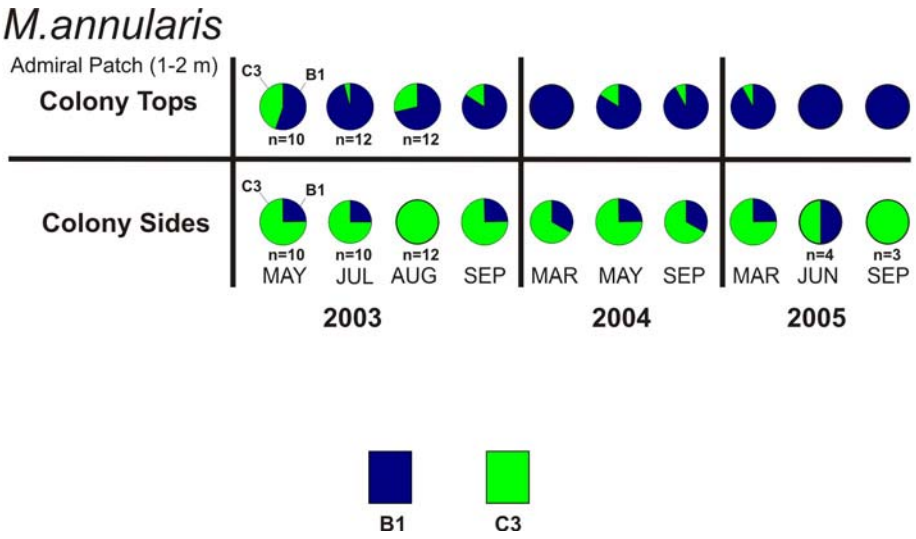


Figure 4.1 *Symbiodinium* ITS 2 types detected in the unshaded colony tops and shaded colony sides of *Montastrea annularis* from the Admiral Patch Reef of the Florida Keys from May 2003 to June 2005. Each pie chart consists of 6 colonies unless otherwise noted by 'n= the number of replicates' below a relevant chart. Appearance of a new symbiont type in each set of colonies is designated with its alpha-numeric clade designation.

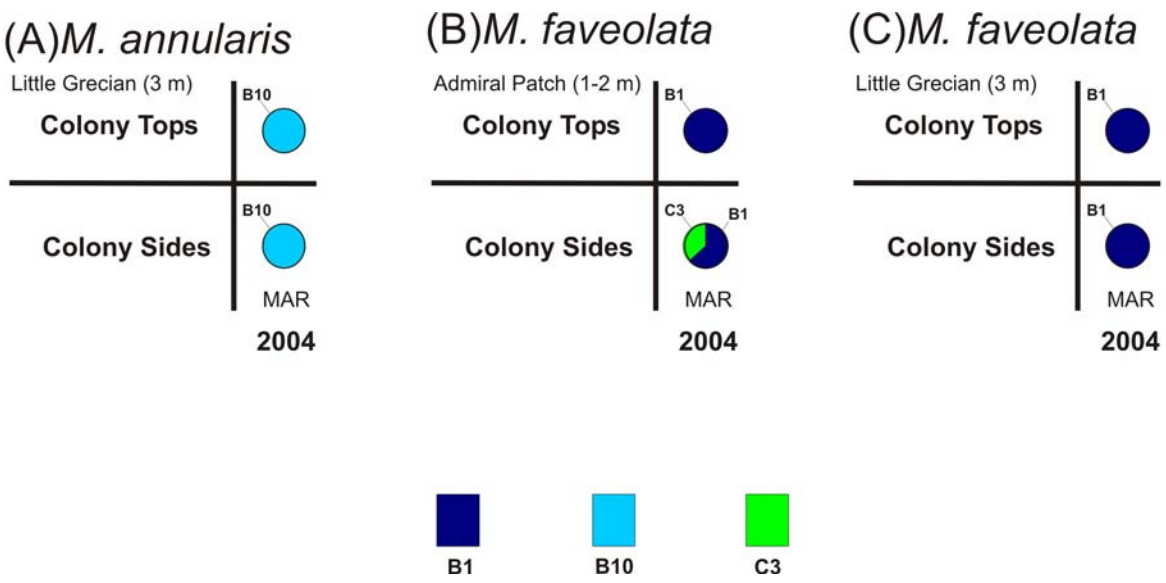


Figure 4.2 *Symbiodinium* ITS 2 types detected in the unshaded colony tops and shaded colony sides of *Montastrea annularis* from the Little Grecian Fore-Reef and *M. faveolata* from both Admiral Patch Reef and Little Grecian Fore-Reef of the Florida Keys. Samples were collected in March 2004. Each pie chart consists of 6 colonies. Appearance of a new symbiont type in each set of colonies is designated with its alpha-numeric clade designation.

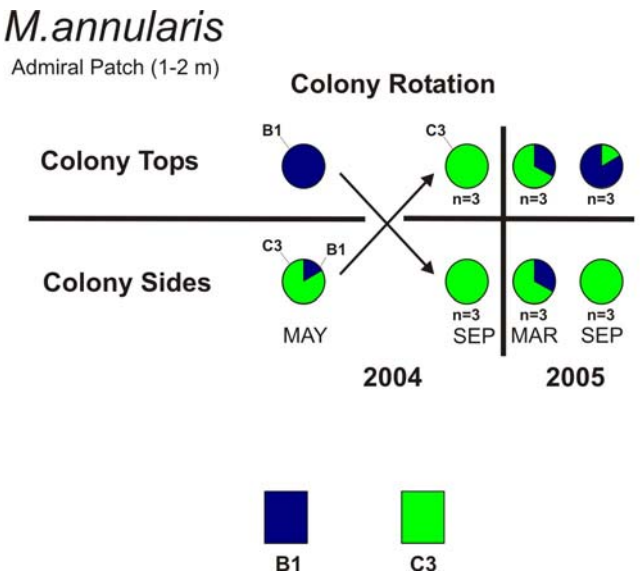


Figure 4.3 *Symbiodinium* ITS 2 types detected in the tops and sides of *Montastrea annularis* colonies before and after rotation. Colonies were rotated 90° such that the tops and sides switched places. Each pie chart consists of 6 colonies unless otherwise noted by 'n= the number of replicates' below a relevant chart. Appearance of a new symbiont type in each set of colonies is designated with its alpha-numeric clade designation.

Figure 4.4 Photograph of rotated *Montastrea annularis* fragment from colony #1 in September 2004. The new colony tops (former colony sides) showed significant visible bleaching as a result of colony rotation.

CHAPTER 5

HIGHLY STABLE SYMBIOSES IN WESTERN ATLANTIC BROODING CORALS[4]

[4] Thornhill DJ, Fitt WK, and GW Schmidt. To be submitted to *Marine Ecological Progress Series*.

Introduction:

The *Symbiodinium* genus of dinoflagellates that associates in mutualisms with scleractinian corals is biochemically, physiologically, and genetically diverse (Trench 1993, 1997; Rowan 1998; LaJeunesse 2001). It consists of 8 major sub-generic clades (A-H)(reviewed in Baker 2003). Within each clade there are numerous more taxonomically specific ITS types with distinctive physiological and ecological attributes (Rowan and Powers 1991; LaJeunesse 2001, 2002; Pochon et al. 2004; Little et al. 2004; Tchernov et al. 2004). A coral colony typically harbors only one detectable type of symbiont, although many coral species associate with more than one symbiont throughout their geographic and depth distributions (Coffroth et al. 2001, Goulet and Coffroth 2003, Iglesias-Prieto et al. 2004, LaJeunesse et al. 2005). Specific host-*Symbiodinium* combinations are highly dependent on the species of coral (LaJeunesse 2002, LaJeunesse et al. 2003), their specific environment (Rowan et al. 1998) and their geographic location (Rodriguez-Lanetty et al. 2001; Loh et al. 2001; van Oppen et al. 2001; LaJeunesse 2002, LaJeunesse et al. 2003, 2004a,b).

The mechanism of symbiont acquisition depends on the reproductive strategy of the coral host (Richmond and Hunter 1990). Most corals broadcast spawn azooxanthellate gametes into the water column (Szmant 1986; Richmond and Hunter 1990). In such cases symbionts must be 'horizontally' acquired from the surrounding environment, thereby creating the possibility for new host-symbiont combinations with each new generation (Szmant 1986; Richmond and Hunter 1990). In contrast corals that internally brooded their larvae transfer symbionts 'vertically' from the maternal parent to offspring (Szmant 1986; Richmond and Hunter 1990). Because of the maternal transmission of zooxanthellae, symbioses in brooding corals are hypothesized to be more specific than those of broadcast spawners (e.g. Loh et al. 2001). Most

available data supports this hypothesis. For instance, Loh et al. (2001) found a higher degree of regional divergence in the symbionts of the brooding *Seriatopora hystrix* compared to the broadcasting *Acropora longicyathus* suggesting regional host-symbiont coevolution and specialization in brooding corals. Across a wide biogeographic gradient, LaJeunesse et al. (2004b) found environment and latitude determined symbiont association in broadcast spawning corals whereas brooding corals associated with a number of closely related symbionts specialized exclusively on a single host genera. Similarly, Barneah et al. (2004) found that symbiont clade correlated with mode of acquisition among Red Sea soft corals. However, other studies found that mode of symbiont acquisition does not affect the overall level of symbiont diversity in *Porites* spp. (Hidaka and Hirose 2000) or Indo-Pacific Acroporid corals (van Oppen, 2004) suggesting that brooding corals are more open to exogenous symbiont infection than previous hypothesized.

Few previous studies have examined multi-year host-symbiont relationships. While available data shows prevalent stable symbioses (Goulet and Coffroth 2003; Stat et al. 2004; Thornhill et al. 2005), fluctuations in *Symbiodinium* spp. populations have been observed in many broadcast spawning corals in response to environmental change (Toller et al. 2001a,b; Thornhill et al. 2005). Hypothetically, brooding corals have more stable symbiotic associations than broadcast spawning corals. To test this hypothesis, we examined the extent to which three species of brooding corals (*Agaricia agaricites*, *Porites astreoides*, and *Siderastrea radians*), which acquire their symbionts from the maternal parent, undergo changes in their symbiotic associations over time. If variance in abiotic factors, such as temperature and light, leads to variable symbioses, this pattern should be evident at the northern and southern limits of reef

occurrence, where seasonality is most pronounced. Therefore, this study examined symbioses in two northern reef systems, the Bahamas and Florida Keys.

Methods:

Coral tissue samples were collected by SCUBA several times per year from three species of brooding scleractinian coral, *Agaricia agaricites* (Linnaeus), *Porites astreoides* (Lamarck), and *Siderastrea radians* (Pallas) from inshore habitat off Lee Stocking Island, the Bahamas (24˚15'N; 76˚30'W) and the Upper Florida Keys (24˚59'N, 80˚22'W). Colonies of *P. astreoides* were sampled from May 2002 to May 2003 in the Bahamas and from May 2002 to September 2005 in the Florida Keys. Colonies of *A. agaricites* and *S. radians* were collected from March 2003 to September 2005. In the Bahamas, colonies of *A. agaricites* and *P. astreoides* were sampled from the inshore North Norman's Patch (2-4m depth). In the Florida Keys, *P. asteroides* was sampled from the inshore Admiral patch reef (1-2m depth), whereas colonies of *S. radians* were sampled from the inshore environment of Florida Bay (1-2m depth).

Six replicate colonies were identified and tagged for each available species to ensure that subsequent collection was from the same colony. *Siderastrea radians* was the only species not collected in this manner due to the small size of colonies; instead samples were obtained from six distinct colonies from approximately the same area each sampling period. Approximately 5-cm diameter tissue samples were collected from 'boulder' corals via hammer and chisel, with care taken to ensure that the same relative position (i.e. the unshaded colony tops) was sampled each time. For 'leaf' colonies of *Agaricia agaricites*, approximately 5-cm diameter leaves were snapped off by hand. Coral fragments were placed in seawater-filled, pre-labeled plastic bags

and transported immediately to the laboratory in an insulated cooler, where they were processed immediately.

Coral tissue was removed from 5 to 25 cm^2 of coral skeleton with a recirculating Water Pik™ using filtered (0.45 μm) seawater. Tissue was removed with this method from equal areas on either side of *Agaricia agaricites* leaves and completely from the tops of *Porites astreoides* and *Siderastrea radians* fragments. This salt-water "blastate" was pulsed briefly (1-4 s) using a Brinkmann Instruments Polytron Kinematica Tissue Homogenizer™ to disperse mucopolysacharides. *Symbiodinium* spp. cells were isolated from the remaining salt-water blastate via centrifugation in 50-ml tubes at 1000 *g* and the resulting algal pellet was preserved in DMSO Buffer (20% dimethyl sulfoxide and 0.25 M ethylene diaminetetraacetic acid (EDTA) in NaCl-saturated water) (Seutin et al. 1991). Samples from 1998 were lyophilized and stored at – 20° C until extraction.

Nucleic acids were extracted using the Wizard® DNA preparation protocol (Promega) following the methods of LaJeunesse et al. (2003), with the following modifications. Most samples of *Porites astreoides* were subjected to a series of washes with 0.01% Triton followed by 3-5 rinses with dH_2O to clean the pellet prior to extraction. In some extractions, 0.3 M Tris, 2 mM EDTA, 0.7% SDS pH 7.5 was used in place of Nuclei Lysis Solution and 7.5 M ammonium acetate was used in place of Protein Precipitation Solution® (Promega).

The internal transcribed spacer 2 region (ITS 2) of nuclear ribosomal RNA was used to discriminate molecular types of *Symbiodinium* (LaJeunesse 2001, 2002). This region was amplified from the DNA extract for denaturing-gradient gel electrophoresis (DGGE) using primers "ITS 2 clamp" (5'CGCCCGCCGC GCCCCGCGCC CGTCCCGCCG CCCCCGCCC GGGATCCATA TGCTTAAGTT CAGCGGT-3') and "ITSintfor 2" (5'GAATTGCAGA

ACTCCGTG-3'). PCR amplification followed the "touchdown" thermal cycle protocol of LaJeunesse (2002). Products of these PCR reactions were checked by electrophoresis on agarose gels (1.0% agarose in 40 mM Tris-acetate, 1mM EDTA solution). Samples containing successfully amplified PCR products were subsequently electrophoresed on denaturing gradient gels (45-80% formamide, 8% polyacrylamide denaturing gradient gels; 100% consists of 7 *M* urea and 40% deionized formamide) and compared to reference samples of known ITS2 type following the protocol of LaJeunesse and Trench (2000), with the modifications of LaJeunesse et al. (2003). Because of difficulty working with extracts of *Porites astreoides* extracts, in addition to DGGE analysis molecular type of *Symbiodinium* was verified via Sanger DNA sequence analysis with an Applied Biodsystems ABI Prism 377^{TM} according to the protocol of LaJeunesse (2002).

Results:

Symbiodinium ITS2 types detected

Three types of *Symbiodinium*, ITS2 types A4a, B5a, and C3a (see LaJeunesse 2002), were detected in the colonies examined in this study. An example PCR-DGGE profile of these three types as well as markers of other common Caribbean *Symbiodinium* spp. is shown (Fig. 5.1).

Patterns in *Agaricia agaricites* symbioses

The symbiotic patterns within coral colonies were tracked between March 2003 and September 2005 for *Agaricia agaricites*. An example diagnostic PCR-DGGE profile of the *Symbiodinium* types detected in one colony of *A. agaricites* is shown (Fig. 5.2). All colonies of *A. agaricites*

were exclusively symbiotic with *Symbiodinium* type C3a, with no mixed symbioses ever detected.

The symbiont types detected in replicate colonies (n=6) of *A. agaricites* are compiled in pie charts from March 2003 to September 2005 (Fig. 5.3). Each pie chart represents one season and a 1/6 wedge of a pie chart designates each colony. Because PCR-DGGE is not a quantitative method, relative abundance above the minimum detection threshold (≥7%, see Thornhill et al. 2005) of a particular symbiont type cannot be precisely determined. As a result, detection of multiple types within a colony is illustrated by splitting a 1/6 wedge between the two types. Each row of pie charts was taken from the same replicate colonies.

At the detection level of DGGE (≥7%, see Thornhill et al. 2005), colonies of *A. agaricites* were invariably symbiotic with *Symbiodinium* type C3a (Figs. 5.2, 5.3). This pattern occurred throughout the sampling; no change was observed in relation to seasonal fluctuations. Additionally, during early September 2005 all *A. agaricites* colonies from the Bahamas experienced significant visual bleaching (Fig 5.4). Despite this, no change in symbiont type was observed.

Patterns in *Porites astreoides* symbioses

Symbiodinium spp. associations with *Porites astreoides* were monitored in six tagged colonies per reef from the Bahamas and Florida Keys seasonally beginning in May 2002. These data were compiled as pie charts as described for *Agaricia agaricites*, with each colony allocated a 1/6 wedge of a chart and each chart representing one time period (Fig. 5.5).

Symbioses in *Porites astreoides* were a stable association with type A4a in all colonies from both the Bahamas and the Florida Keys (Fig. 5.5). Individual colonies showed fidelity over

time to one particular *Symbiodinium* spp. partner and changing symbiont types was non-existent in all colonies sampled.

Patterns in *Siderastrea radians* symbioses

Siderastrea radians symbioses were monitored beginning in March of 2003 from non-tagged colonies of the same area of Florida Bay. Pie charts representing *S. radians* symbiotic associations were compiled in the same method described for *Agaricia agaricites* symbioses, with each colony allocated a 1/6 wedge of the chart (Fig. 5.2). *Symbiodinium* type B5, a specialist symbiont in the genus *Siderastrea* (LaJeunesse 2002) was the only detected symbiont in *S. radians* (Fig. 5.6). *Siderastrea radians* associations unchanged from March 2003 to September 2005. Additionally, during March of 2003 colonies varied significantly in overall appearance, with some colonies appearing extremely dark brown and others appearing bleached to light pink. Regardless of the colony color, all colonies were detected in association with only type B5 (Table 5.1).

Discussion:

At the detection level of DGGE (≥7%, see Thornhill et al. 2005), all brooding coral colonies examined were absolutely invariable in their symbiotic associations with *Symbiodinium* spp. This stability exceeds that encountered for most broadcast spawning corals sampled from the same location and time (see Thornhill et al. 2005). Although most broadcast spawning colonies were markedly stable in their symbioses, occasional introgressions occurred wherein mixed populations of multiple ITS2 types were detected (Thornhill et al. 2005). No such mixed populations occurred for any of the brooding colonies examined.

Such invariable symbioses in brooding corals are most easily explained by the reproductive mode of the host. Vertical transmission of symbionts between the maternal parent and offspring limits the opportunity for the intergenerational symbiont change. Hypothetically, this would result in a more tightly coevolved relationship between host and symbiont, thereby reducing the opportunity for symbiotic change (Douglas 1998; Loh et al. 2001, Rodriguez-Lanetty et al. 2001). Indeed, *Symbiodinium* types C3a and B5a are considered specialist types found only in their respective host genus (C3a in *Agaricia* spp. and B5a in *Siderastrea* spp.) (LaJeunesse 2002). Similar results are described for *Symbiodinium* types (C21 and related types) associating exclusively with brooding *Montipora* spp. corals (LaJeunesse et al. 2004b).

Reefs of the Bahamas and Florida Keys are approaching the northern limits of reef accretion where seasonal fluctuations in temperature and light are most pronounced. Despite the intensity of these seasonal changes and a bleaching event in the Bahamas, no change in symbiont type was observed among any of the brooding corals examined. Barneah et al. (2004) suggested that the coevolved specialist symbionts found in brooding colonies must be able to tolerate a brood range of environmental conditions. The results of this study, compared with previous work on broadcast spawning colonies (Thornhill et al. 2005) points to a similar conclusion. Change in the dominant type of *Symbiodinium* is hypothesized to facilitate acclimatization to a broad range of environmental conditions (Rowan and Powers 1991; Buddemeier and Fautin 1993; LaJeunesse 2005). Corals that maintain highly stable symbiotic associations with one dominant type of *Symbiodinium* through a range of environmental conditions must be capable of responding to environmental change through acclimatization or adaptation on the part of either the coral host and/or the dinoflagellate symbiont.

Porites astreoides is known to be symbiotic with several different ITS types of *Symbiodinium* (including types A4a, A3, and B1, LaJeunesse 2002), however colonies from this from this study were detected with only type A4a. It is possible that there are distinct subpopulations of *P. astreoides* and other brooding corals which each associate with a distinct maternally inherited *Symbiodinium* type. Comparative analysis of the host and symbiont population structure is necessary to test this hypothesis. Previous analyses of broad biogeographic patterns suggest that this is the case (Loh et al. 2001; LaJeunesse et al. 2004b), however brooding corals show similar level of overall symbiont diversity to broadcast spawners (Hidaka and Hirose, 2000; van Oppen 2004). Additionally, Baker (2001) found that in *P. astreoides* colonies experimentally transplanted from deep to shallow habitats, colonies of (n= 5 out of 8) changed their dominant symbiont from clade C to clade A *Symbiodinium*, thereby demonstrating that *P. astreoides* colonies are not fixed in their symbioses. Whether changes in the dominant type of *Symbiodinium* are a result of the up regulation of a pre-existing (maternally inherited) endogenous type or the acquisition of a new exogenous type remains an open question (see also Lewis and Coffroth 2004).

The overall trend of highly stable symbioses (Goulet and Coffroth 2003; Thornhill et al. 2005), even through bleaching events (Stat et al. 2004; Thornhill et al. 2005; this study), appears to be especially true for brooding corals. Whether symbiotic change occurs in brooding colonies through the predicted rise in global sea-surface temperature (Hoegh-Guldberg 1999; Hughes et al. 2003) remains an open question. Future experiments should focus on the viability and physiological capacity of various host – symbiont combinations in order to predict how coral symbioses will respond to climatic change.

Table 5.1 *Symbiodinium* ITS 2 type detected in dark-brown and light-pink color morphs of *Siderastrea radians* from Florida Bay March 2003.

S. radians Holobiont Color	*N*	Symbiont ITS2 Type
Brown	5	B5a
Light-Pink (bleached)	5	B5a

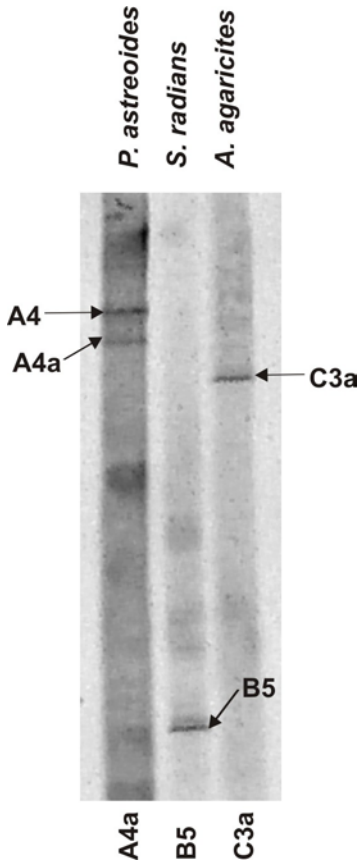


Figure 5.1 Example PCR-DGGE profile of the *Symbiodinium* ITS 2 region for all *Symbiodinium* types detected in this study. Diagnostic band labeled for types A4, C3a, and B5. Faint, repeated bands in each profile are possibly rare intragenomic variants and heteroduplexes created between them and dominant sequences.

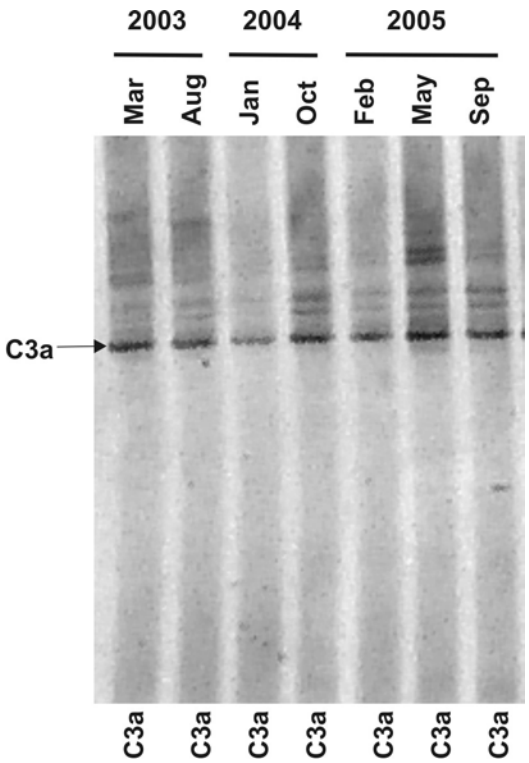


Figure 5.2 PCR-DGGE profile of the *Symbiodinium* ITS 2 region collected from a single colony (colony #1) of *Agaricia agaricites* from North Norman's Patch reef (depth 3 m) in the Bahamas. Gel profile tracks this colony's symbiosis from March 2003 to September 2005 and is presented as a reverse image. Diagnostic band labeled for type C3a. Faint, repeated bands in each profile are possibly rare intragenomic variants and heteroduplexes created between them and dominant C3 and C3a sequences.

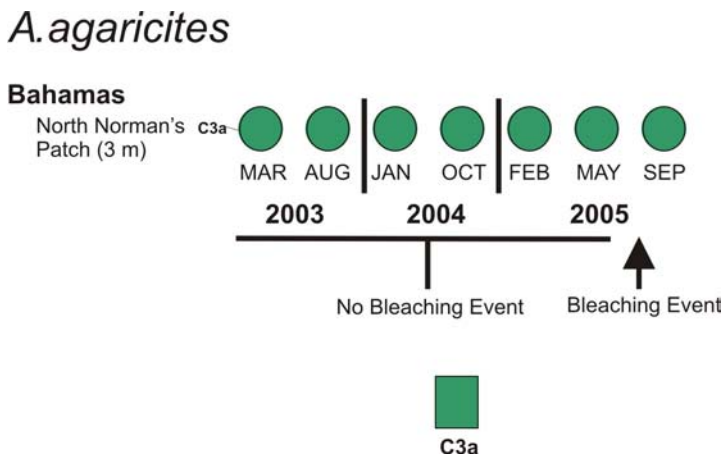


Figure 5.3 *Agaricia agaricites* ITS 2 *Symbiodinium* types detected in colonies (n=6) from the Bahamas March 2003 to September 2005. Symbiont type in each set of colonies is designated with its alpha-numeric clade designation. Bleaching history of these colonies is listed below the relavant pie charts.

Figure 5.4 *A. agaricites* colony from North Norman's Patch Reef off Lee Stocking Island in the Bahamas exhibiting significant visual bleaching in September 2005. Photo by D. Kemp.

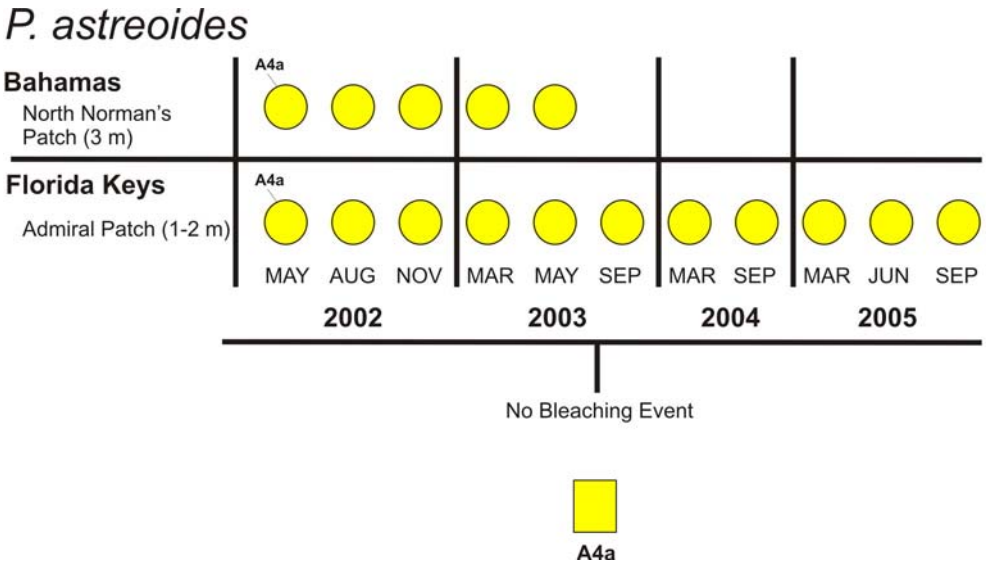


Figure 5.5 *Porites astreoides* ITS 2 *Symbiodinium* types detected in colonies from the Bahamas and Florida Keys from March 2002 to September 2005. Colonies (n=6) listed by region, reef type, and depth as rows of pie charts. Occasionally, fewer than 6 replicates were available. These instances are demarcated by 'n= the number of replicates' below a relevant chart. Symbiont type in each set of colonies is designated with its alpha-numeric clade designation.

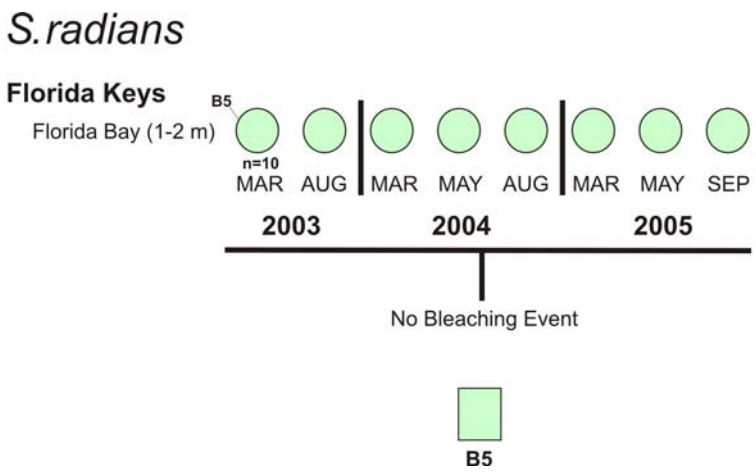


Figure 5.6 *Siderastrea radian* ITS 2 *Symbiodinium* types detected in colonies (n=6) from the Florida Keys March 2003 to September 2005. Symbiont type in each set of colonies is designated with its alpha-numeric clade designation. Instances of greater than 6 colonies examined are demarcated by 'n= the number of replicates' below a relevant chart.

CHAPTER 6

SEASONAL PATTERNS OF HOLOBIONT PHYSIOLOGY FROM THE CARIBBEAN REEF-BUILDING CORAL *MONTASTREA ANNULARIS* IN RELATION TO ITS DOMINANT SYMBIODINIUM SPP. TYPE[5]

[5] Thornhill DJ, Pate W, Todd BT, vanWoesik R, Fitt WK, and GW Schmidt. To be submitted *to Limnology and Oceanography*.

Introduction:

Research on coral-dinoflagellate symbiosis has recently focused on how different *Symbiodinium* spp. types influence the overall physiology of the host-symbiont combination (holobiont). *Symbiodinium* is now recognized as a genetically diverse lineage, including at least eight sub-generic clades (A-H) (reviewed in Baker 2003) and numerous more specific "species" types (LaJeunesse 2001). While it has been widely hypothesized that the lineage of symbiont significantly impacts the physiology of the holobiont (Buddemeier and Fautin 1993; LaJeunesse 2005), relatively few studies have addressed this question directly. For instance, Little et al. (2004) showed that symbiont type had a highly significant effect on the colony growth rate in two species of *Acropora* from the Great Barrier Reef (GBR); colonies harboring clade C *Symbiodinium* grew two to three times faster than those harboring clade D.

Thermal tolerance in *Symbiodinium* spp. is of particular interest because of growing concern over the temperature and light induced bleaching (reviewed in Lesser 2004). LaJeunesse et al. (2003) found that bleaching-resistant *Montipora digitata* from the GBR harbored ITS type C15 Symbiodinium, whereas thermally sensitive *Montipora* spp. harbored other C types. Clade D is widely reported as imparting thermal tolerance because of its presence in colonies from high temperature environments (Toller et al. 2001a; van Oppen et al. 2001) as well as in colonies recovering from bleaching (Toller et al. 2001b; Baker et al. 2004). Rowan (2004) showed that *Pocillopora* spp. corals harboring *Symbiodinium* clade D maintained or even improved their photosynthetic capacity of PSII and maximum net photosynthesis when subjected to increasing temperature. Similarly treated *Pocillopora* spp. harboring clade C were temperature sensitive, experiencing chronic photoinhibition and decreases in maximum net photosynthetic rate (Rowan 2004).

In most coral species, the dominant *Symbiodinium* spp. type does not appear to change seasonally (Goulet and Coffroth 2003; Thornhill et al. 2005; but see Chen et al. 2005). However, scleractinian corals and their symbiotic dinoflagellates (zooxanthellae) do experience significant seasonal fluctuations in many physiological parameters. For instance, zooxanthellae undergo regular seasonal changes in density, a phenomenon believed to be ubiquitous among symbiotic corals (Muller-Parker 1987; Stimson 1997; Brown et al. 1999; Fagoonee et al. 1999; Fitt et al. 2000). Seasonal highs in zooxanthellae density typically occur during the winter months when temperature and irradiance are at the lowest annual levels (Fagoonee et al. 1999; Fitt et al. 2000). Similar fluctuations occur in the pigment content (chlorophyll-a) (Brown 1999; Fitt et al. 2000; Warner et al. 2002) and photosynthetic capacity of PSII (Warner et al. 2002), with circannual maxima occurring during the winter months. Coral tissue biomass (as measured by ash-free dry weight) likewise changes seasonally (Fitt et al. 2000; Warner et al. 2002). However peak tissue biomass occurs in the spring after peaks in zooxanthellae density, suggesting that that there is a functional relationship between these two parameters (Fitt et al. 2000). Corals living in shallow water usually have higher levels and more significant fluctuations of all these parameters (except chlorophyll-a) (Fitt et al. 2000).

Nothing is currently known about how *Symbiodinium* spp. type influences seasonal fluctuations in the physiology of the holobiont. The availability of molecular tools to study *Symbiodinium* diversity (Rowan and Powers 1991) has enabled the in depth investigation of the degree to which coral-algal symbioses undergo changes in partner association from season to season and year to year (Thornhill et al. 2005). This study utilizes a natural experiment, initially documented in Thornhill et al. (2005), where shallow colonies of the coral *Montastrea annularis* experienced different patterns of symbiotic association in the Florida Keys than in the Bahamas

from March 2000 to May 2002. *M. annularis* from Florida harbored mixed communities of *Symbiodinium* spp., including the putatively stress tolerant clade D, which were attributed to the 1997-1998 bleaching event. Clade D *Symbiodinium* was eventually displaced from the Florida *M. annularis* colonies and subsequently (August 2002 to August 2004) colonies from both regions experienced stable symbioses with clade B *Symbiondinium* spp. Comparisons of these two reef systems while there symbioses were behaving differently (March 2000 to May 2002) and when they were similar (August 2002 to August 2004) may provide insight into the influence of symbiont type on holobiont physiology.

Methods:

Coral tissue samples were collected by SCUBA quarterly (March, May, August, and November) from the scleractinian coral *Montastrea annularis* (Ellis and Solander) from Little Grecian Reef (3-4 m depth) off of the Upper Florida Keys (24˚59'N, 80˚22'W) and North Norman's Patch Reef (2-4 m depth) off Lee Stocking Island, the Bahamas (24˚15'N; 76˚30'W). Coral colonies were sampled regularly between March 2000 and August 2004. Six replicate colonies were identified and tagged to ensure that subsequent collection was from the same colony. Approximately 25-50cm^2 tissue samples were collected via hammer and chisel, with care taken to ensure that the same relative position (i.e. the unshaded colony tops) was sampled each time. When possible, tissue fragments were split into two halves; otherwise replicate samples were collected from each colony. Coral fragments were placed in individual seawater-filled, pre-labeled plastic bags and transported immediately to the laboratory in an insulated cooler, where they were processed immediately.

Upon return to the laboratory, the one replicate coral fragment was placed in a dish with seawater and kept in the dark for 30 min. Following this dark acclimation, samples were placed under a dark cloth and readings were measured using a diving pulse amplitude modulated (PAM) fluorometer (Walz, Germany). Chlorophyll fluorescence was measured using by recording the minimum (F_0) and maximum (F_m) fluorescence with a diving PAM fluorometer as described by Warner et al. (1996). The photosynthetic capacity of PSII or quantum yield of PSII charge separation (F_v/F_m) was then calculated as: $(F_m-F_0)/F_m$.

Following measurement of the photosynthetic capacity, tissue was removed from the replicate coral fragments with a recirculating Water Pik™ using filtered (0.45 µm) seawater. The salt-water "blastate" was pulsed briefly (1-4 s) using a Brinkmann Instruments Polytron Kinematica Tissue Homogenizer™ to disperse mucopolysacharides. The total resulting homogenate volume was then measured and a 1-ml aliquot was taken and preserved with 1 drop of 40% formalin for cell counts. Densities of zooxanthellae were measured from replicate (n=8) hemocytometer counts. Two additional 15-ml subsamples were taken for chlorophyll-a (Chl-a) analysis. Chlorophyll samples were centrifuged for 5 min at 2000 *g* and the remaining pellet was immediately frozen at –20 ºC to assist in breaking open the cells. Acetone was added to the frozen pellet and the resulting mixture (~90% Acetone) was homogenized in a glass tissue grinder and the homogenate was allowed to develop overnight in the dark at –20 ºC. Absorbances were measured using a spectrophotometer and chlorophyll-a content was calculated by the equations of Jeffery and Humphrey (1975). Surface area of was determined by cutting aluminum foil pieces to precisely cover the area of tissue-removed coral skeleton. Correlations between weight and surface area of aluminum foil enabled the calculation of zooxanthellae and chlorophyll densities.

Symbiodinium spp. cells were isolated from the remaining salt-water blastate via centrifugation in 50-ml tubes at 2000 *g* and the resulting algal pellet was preserved in DMSO Buffer (20% dimethyl sulfoxide and 0.25 M ethylene diaminetetraacetic acid (EDTA) in NaCl-saturated water) (Seutin et al. 1991). Samples from 1998 were lyophilized and stored at –20° C until extraction.

Nucleic acids were extracted using the Wizard® DNA preparation protocol (Promega) following the methods of LaJeunesse et al. (2003). In some extractions, 0.3 M Tris, 2 mM EDTA, 0.7% SDS pH 7.5 was used in place of Nuclei Lysis Solution. RNA digestion in all extractions was by 10mg ml-1 RNAse A for 30 min at 37º C. Also, in some extractions 7.5 M ammonium acetate was used in place of Protein Precipitation Solution® (Promega).

The internal transcribed spacer 2 region (ITS 2) of nuclear ribosomal RNA was used to discriminate molecular types of *Symbiodinium* (LaJeunesse 2001, 2002). This region was amplified from the DNA extract for denaturing-gradient gel electrophoresis (DGGE) using primers "ITS 2 clamp" (5'CGCCCGCCGC GCCCCGCGCC CGTCCCGCCG CCCCCGCCC GGGATCCATA TGCTTAAGTT CAGCGGT-3') and "ITSintfor 2" (5'GAATTGCAGA ACTCCGTG-3'). PCR amplification followed the "touchdown" thermal cycle protocol of LaJeunesse (2002). Products of these PCR reactions were checked by electrophoresis on agarose gels (1.0% agarose in 40 mM Tris-acetate, 1mM EDTA solution). Samples containing successfully amplified PCR products were subsequently electrophoresed on denaturing gradient gels (45-80% formamide, 8% polyacrylamide denaturing gradient gels; 100% consists of 7 *M* urea and 40% deionized formamide) and compared to reference samples of known ITS2 type following the protocol of LaJeunesse and Trench (2000), with the modifications of LaJeunesse et al. (2003).

Tissue was completely removed from 5 to 25 cm^2 of the second of the replicate coral fragments with a recirculating Water Pik™ using distilled water. This freshwater "blastate" was immediately frozen at –20 ºC. Frozen samples were later lyophilized prior to dry weight analysis. Dry weight was determined for three replicate subsamples of lyophilized homogenate before ashing in a Fisher Scientific muffle furnace at 500 ºC. Ash-free dry weight was determined by calculating the difference between dry weight of lyophilized homogenate and ash weight. Surface area was determined by cutting aluminum foil pieces to precisely cover the area of tissue-removed coral skeleton. Correlations between weight and surface area of aluminum foil enabled the calculation of ash-free dry weight cm^{-2}.

Temperature data were obtained from instrument data available publicly through the NOAA National Buoy Center (http://www.ndbc.noaa.gov/) for sea surface temperatures at Molasses Reef and NOAA/Caribbean Marine Research Center (CMRC) sea surface temperatures at North Norman's Patch Reef (ftp://data.nodc.noaa.gov/pub/outgoing/CoRIS/data/pims/watertempsBahamas/).

All numerical data were tested for assumptions of normality and homogeneity of variance. Significant differences were determined by repeated measures analysis of variance (ANOVA, Statistica 6) at a $p < 0.05$ level of confidence. All data are presented graphically in terms of means ± SE.

Results:

Symbiodinium spp. type

The symbiont types detected in replicate colonies (n=6) of *Montastrea annularis* are compiled in pie charts from March 2000 to August 2004 (Fig. 6.1). Each pie chart represents

one season and a 1/6 wedge of a pie chart designates each colony. Because PCR-DGGE is not a quantitative method, relative abundance above the minimum detection threshold (≥7%, Thornhill et al. *in press*) of a particular symbiont type cannot be precisely determined. As a result, detection of multiple types within a colony is illustrated by splitting a 1/6 wedge between the two types. Each row of pie charts was taken from the same replicate colonies.

Colonies of *Montastrea annularis* from the Florida Keys (Little Grecian fore reef at 3 m) experienced changes in the symbiont partner that did not occur in the Bahamas (Fig. 6.1). Sampling from tagged colonies revealed temporal changes in the identity and proportion of symbiont types D1a, B1, and B10 (Little Grecian reef) from March 2000 until May 2002, after which a stable dominance of type B10 (Little Grecian reef) was reached, four to five years after the 1997-8 El Niño episode ended (Fig. 6.1). *M. annularis* from the Bahamas had invariable symbioses with a monotypic population of *Symbiodinium* type B1 for all times measured (Fig. 6.1). These data are part of an ongoing survey; see Thornhill et al. (2005) for the complete data set.

Density of symbiotic dinoflagellates

Seasonal fluctuations of zooxanthellae densities from both reefs are shown (Fig. 6.2). From March 2000 to May 2002 on Little Grecian fore reef, when *M. annularis* experienced changes in their dominant type of *Symbiodinium* (Fig. 6.1), zooxanthellae densities fluctuated seasonally, with lowest densities typically occurring during the late summer (but also the late winter of 2001) (Fig. 6.2). During that same period on North Norman's Patch reef, which did not experience changes in the dominant type of *Symbiodinium*, the seasonal trend was less pronounced, with the lowest densities occurring during the late winter or early summer (Fig.

6.2). These seasonal fluctuations were significant over time for both reefs ($p < 0.05$). From March 2000 to May 2002, densities of symbiotic dinoflagellates were overall significantly ($p < 0.000001$) higher on Little Grecian fore reef than on North Norman's Patch reef. Additionally during this same period, the site*time interaction was significantly ($p < 0.05$) different indicating that the seasonal pattern of each reef were different (Fig. 6.2).

From August 2002 to August 2004, when corals from both reefs harbored stable populations of clade B *Symbiodinium* (Fig. 6.2), no significant seasonal fluctuations occurred on either reef ($p > 0.05$). The Little Grecian for reef no longer had significantly higher densities of symbiotic dinoflagellates ($p > 0.05$) and there was no significant site*time interaction difference between reefs ($p > 0.05$) suggesting that these two reefs behaved similarly overall during this time.

Pigment Density and Content

Mean Chlorophyll-a densities for each reef are shown (Fig 6.3). Corals of Little Grecian fore reef experienced higher overall density of chlorophyll-a than those of North Norman's Patch reef from both March 2000 to May 2002 when mixed symbiont assemblages occurred on Little Grecian ($p < 0.00001$) and from August 2002 to August 2004 ($p < 0.005$) when the symbiont community was stable on both reefs (Fig. 6.1). There was significant seasonal variation in chlorophyll-a density from March 2000 to May 2002 on both reefs ($p < 0.0001$) with the highest overall levels occurring in the late fall. This seasonal pattern was not subsequently apparent from August 2002 to August 2004 on either reef ($p > 0.05$). Overall, there was no significant site*time interaction difference for either the March 2000 to May 2002 ($p > 0.05$) or August

2002 to August 2004 ($p > 0.05$) suggesting that trend of chlorophyll density fluctuation these reefs was similar overall regardless of symbiont type.

There was a significantly positive correlation between density of chlorophyll-a and symbiont density ($p < 0.0001$, $r^2 = 0.385$; Fig. 6.4). However, chlorophyll-a density did not increase as rapidly as zooxanthellae density (Fig. 6.4). Some of this disparity can be explained by acclimatization on the part of the zooxanthellae, as there was a significant decrease in chlorophyll-a per cell as zooxanthellae density increased ($p < 0.001$, $r^2 = 0.114$; Fig. 6.5).

Photosynthetic capacity of PSII

There was significant seasonal variation in the photosynthetic capacity of PSII (F_v/F_m) for coral colonies from both reefs ($p < 0.000001$; Fig. 6.6) throughout this study, including from March 2000 to May 2002 ($p < 0.0001$) as well as from August 2002 to August 2003 ($p = 0.000001$). *M. annularis* from Little Grecian reef experienced highest F_v/F_m values during the late summer of 2000 and 2001, when mixed populations of *Symbiodinium* types B1, B10 and D1a were present (Fig. 6.1). In contrast, colonies from North Norman's Patch reef, which maintained stable associations with type B1 symbionts, experienced peak F_v/F_m values during the late winter of 2000 and 2001 (Fig 6.6). There was a highly significant time*site interaction effect during this time ($p < 0.000001$) indicating that the seasonal pattern in photosynthetic capacity was dissimilar on two reefs during the first half of this study (Fig. 6.6).

The patterns of photosynthetic capacity of PSII between Little Grecian and North Norman's Patch became more similar beginning in May of 2002, with highest F_v/F_m values occurring during the early or late winter at both sites (Fig 6.6). However, the time*site

interaction effect from August 2002 to August 2003 was still significantly different between sites ($p < 0.001$).

Coral tissue biomass

Mean coral tissue biomass, represented as ash-free dry weight, is show for Little Grecian and North Norman's Patch reefs from March 2000 to August 2004 (Fig. 6.7). During the period of mixed symbiont assemblages on Little Grecian reef, March 2000 to May 2002 (Fig. 6.7), Little Grecian *M. annularis* colonies experienced significant seasonal fluctuations in tissue biomass ($p < 0.05$), with peaks most often pronounced in early to late summer (Fig. 6.7). North Norman's Patch, which didn't experience mixed symbiont assemblages, also had significant ($p < 0.05$) seasonal differences, with highs typically in the late winter to early summer from 2000 to 2002. While the average tissue biomass was not significantly different between sites ($p > 0.05$) during March 2000 to May 2002, there were significant site*time interaction differences ($p < 0.005$) indicating that these reefs were out of seasonal phase with one another (Fig. 6.7).

Significant site*time interaction differences in tissue biomass disappeared during August 2002 to August 2004 when corals from both reefs harbored a stable community of clade B *Symbiodinium* ($p > 0.05$). Fluctuations in tissue biomass were less extreme on both reefs during this 2002 to 2004 period. However a significant seasonal pattern still remained ($p > 0.05$) with slight peaks tending to occur more in November or March. No difference in overall tissue biomass averages occurred between sites during this time ($p > 0.05$).

Sea surface temperature

Hourly sea surface temperature (Fig 6.8) and mean daily sea surface temperature (Fig 6.9) from October 1999 to April 2003 for Molasses Reef of the Upper Florida Keys and North Norman's Patch Reef in the Bahamas. Temperature profiles for these two regions were generally similar. However, seasonal temperature fluctuations were more extreme in the Florida Keys than in the Bahamas, particularly during the winter months.

Discussion:

This study is the first to show seasonal differences in the physiology of the coral-dinoflagellate holobiont related to the genetic identity of *Symbiodinium*. *Montastrea annularis* colonies from Little Grecian fore reef harbored mixed populations of *Symbiodinium* types B10, B1 and D1a from March 2000 to May 2002, while colonies from North Norman's Patch reef harbored stable populations of type B1. During this time corals of Little Grecian reef had higher average densities of zooxanthellae and chlorophyll-a, and had significantly different seasonal patterns in zooxanthellae density, photosynthetic capacity of PSII and coral tissue biomass than did corals from North Norman's Patch. When the symbiont community subsequently stabilized to a monotypic community of type B10 on Little Grecian fore reef, similar to the monotypic community of type B1 on North Norman's Patch, no differences were observed between reefs in the average zooxanthellae density or seasonal patterns in fluctuation of zooxanthellae density and coral tissue biomass. Additionally, differences in the seasonal fluctuation of photosynthetic capacity of PSII became less significant. This pattern supports previous studies indicating that a colony's symbiont community has a significant effect on holobiont physiology (e.g. Little et al.

2004; Rowan 2004), a key assumption of the adaptive bleaching hypothesis (ABH) (Buddemeier and Fautin 1993).

Regional differences in temperature and light may be driving the different symbiont communities encountered in *M. annularis* colonies in this study (see also Thornhill et al. 2005) and may be affecting other physiological variables as well. The greater temperature fluctuations, including more severe winter lows, experienced in the Florida Keys may help determine the dominant population of *Symbiodinium* spp. (see also Toller et al. 2001a). Kemp (2003) observed a similar phenomenon and demonstrated that locations with greater temperature fluctuations experienced a lower degree of bleaching in the zoanthid *Palythoa caribaeorum*. All the colonies examined in this study bleached during the 1997-98 El Niño Southern Oscillation (ENSO) event and regional differences in bleaching intensity or symbiont availability may explain the symbiont community differences (Thornhill et al. 2005). Additionally, regional differences in light can be inferred through the consistently higher chlorophyll density on Little Grecian reef. This pattern indicates that colonies from Florida experienced lower overall light intensities throughout this study, perhaps as a result of latitude or decreased water clarity from nutrient enrichment (Lapointe and Clarke 1992). It is interesting to note that these two reefs showed remarkable similar trends in their seasonal fluctuations of chlorophyll density, suggesting that generally similar environmental conditions are experienced on both reefs, albeit with different intensities.

In many respects, the physiological seasonal fluctuations from 2000 to 2002 on Little Grecian Reef are out of phase with the patterns previously documented in the published literature. Fitt et al. (2000) and Warner et al. (2002) showed that for many species of coral, including *Montastrea annularis*, highs in zooxanthellae density, pigment content and photosynthetic capacity of PSII typically occurred during the winter months when temperature

and light are at annual lows. During the 2000 to 2002 time period, Little Grecian experienced seasonal lows in zooxanthellae density in the late winter and late summer months. Additionally, the peaks in F_v/F_m on Little Grecian reef occurred in the summer months during the annual highs in temperature and light, the opposite of the pattern documented by Warner et al. (2002). This difference in phase may be attributable to the presence mixed symbiont assemblages on Little Grecian, in particular the presence the stress tolerant symbiont type D1a (Baker 2003). Rowan (2004) found that a type of clade D imparted greater photosynthetic capacity of PSII (F_v/F_m) and maximum net photosynthetic rate in *Pocillopora* spp. corals under increased thermal stress. The presence of type D1a on Little Grecian reef may help to explain the summer peaks in F_v/F_m. Furthermore the low F_v/F_m values and zooxanthellae densities observed in the winter and early summer of 2000 and 2001, may indicate that type D1a's lower thermal limits are being approached during the coldest months of the year, as has been observed for certain vascular plants (Westin et al. 1995; Herzka and Dunton 1997). The patterns of photosynthetic capacity of PSII are closer to expected seasonal values on North Norman's Patch reef from 2000 to 2002 and on both reefs from 2002 to 2004 when stable symbiont communities are present.

Previous studies found that coral tissue biomass increases to annual highs in the winter or spring following, and perhaps related to, the seasonal cycling of symbiont density (Fitt et al. 2000; Warner et al. 2002). While this was largely true of colonies from North Norman's Patch (2000 to 2004) and colonies of Little Grecian with stable symbioses (August 2002 to August 2004), it was not the case for the Little Grecian harboring mixed symbioses (2000 to 2002). Instead, these colonies reached seasonal maxima in early to late summer. This result may be noteworthy considering the important role that tissue thickness may play in surviving bleaching events (Loya et al. 2001). Hoegh-Guldburg (1999) predicted that thicker tissues would protect

corals from bleaching by photo-protective shielding from excess light. Brown et al. (1997) hypothesized that expansion and retraction of tissue would provide a rapid means of adjusting the intensity of light energy reaching zooxanthellae. When tissues are retracted zooxanthellae would be self-shaded (Brown et al. 1997). Presumably, the capacity for this rapid acclimatization would be greatest in thicker tissue colonies. Furthermore, Loya et al. (2001) demonstrated that following bleaching "winning" coral species had both thick tissue and a massive or encrusting morphology. Therefore unusually high summer tissue biomass in Little Grecian colonies (2000 to 2002) may help protect these corals from the circannual maxima in temperature and light and is possibly related to the presence of the stress-tolerant symbiont type D1a.

One difficulty encountered in this experimental approach is the abundance of mixed symbiont communities, including both type B10 and D1a, during the 2000 to 2002 time period on Little Grecian reef. This considerable mixing combined with little information about the relative abundances of different symbiont types within colonies (Thornhill et al. 2005) makes interpretation of these data difficult. Despite these limitations, significant differences were encountered between reefs during the 2000 to 2002 period that were not apparent during the 2002 to 2004 period indicating that either the presence of a mixed symbiont community or of novel symbiont types (D1a) has a significant impact on holobiont physiology.

The adaptive bleaching hypothesis (ABH) predicts that corals may be able to acclimatize to changes in their environment through change in their dominant lineage of symbionts (Buddemeier and Fautin 1993). Implicit in this hypothesis is the assumption that holobiont physiology changes when corals associate with different types of *Symbiodinium* spp. An alternative hypothesis has recently been offered, contended that species are 'neutral' or identical

in their competitive ability and other properties (Volkov et al. 2003; but see discussion in Volkov et al. 2004). Previous work on *Symbiodinium* physiology suggests that symbionts do vary in both their competitive ability (Fitt 1984; Belda-Baillie et al. 2002) as well as temperature tolerance (Warner et al. 1999; Kinzie et al. 2001; Rowan 2004; Tchernov et al. 2004). However, harboring thermal tolerant symbionts (such as member of clade D) may result in a trade off in terms of colony growth (Little et al. 2004). Additionally, thermal tolerant symbionts may not be equally competitive under non-stress conditions (as was hypothesized in Thornhill et al. 2005). This study suggests the dominant type of *Symbiodinium* sp. has significant effects on physiology of the holobiont from similar environments.

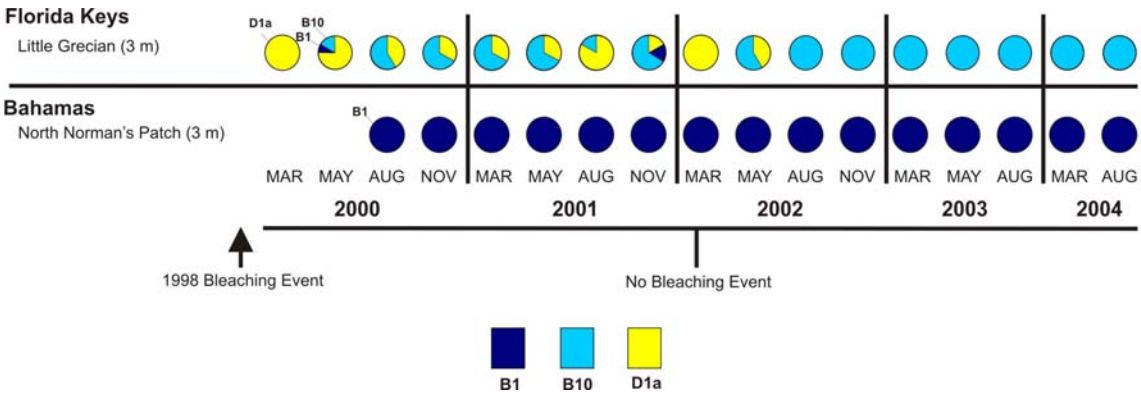


Figure 6.1 *Montastrea annularis* ITS 2 *Symbiodinium* types detected in colonies from the Bahamas and Florida Keys from March 2000 to August 2004. Colonies (n=6) are listed by region, reef type, and depth as rows of pie charts. Appearance of a new symbiont type in each set of colonies is designated with its alpha-numeric name

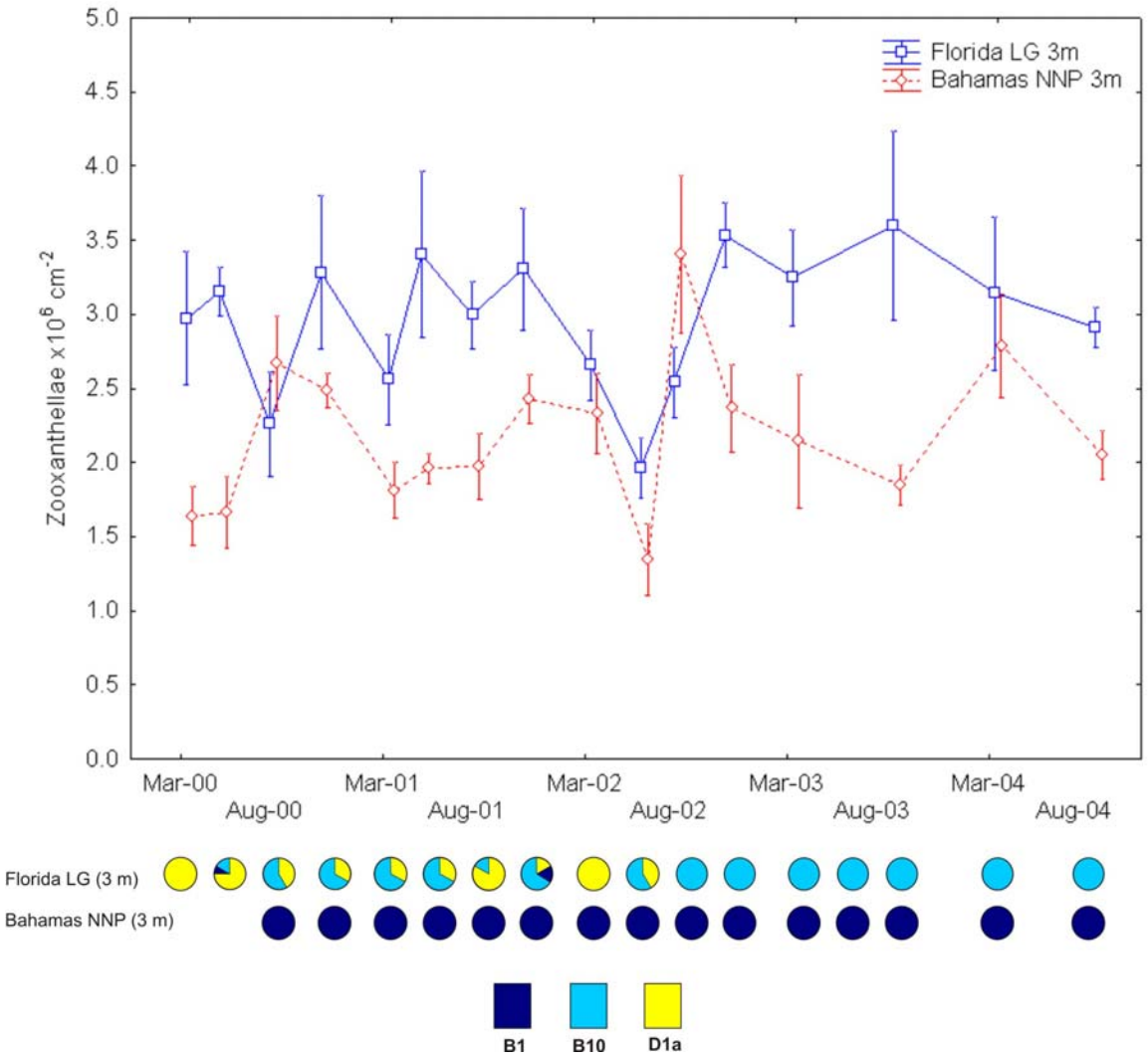


Figure 6.2: *Montastrea annularis* zooxanthellae density in relation to time of year from two shallow (3m) reefs of the Florida Keys and Bahamas. Data are expressed as mean ± SE. *Symbiodinium* spp. type data (Fig. 5.1) is included for reference.

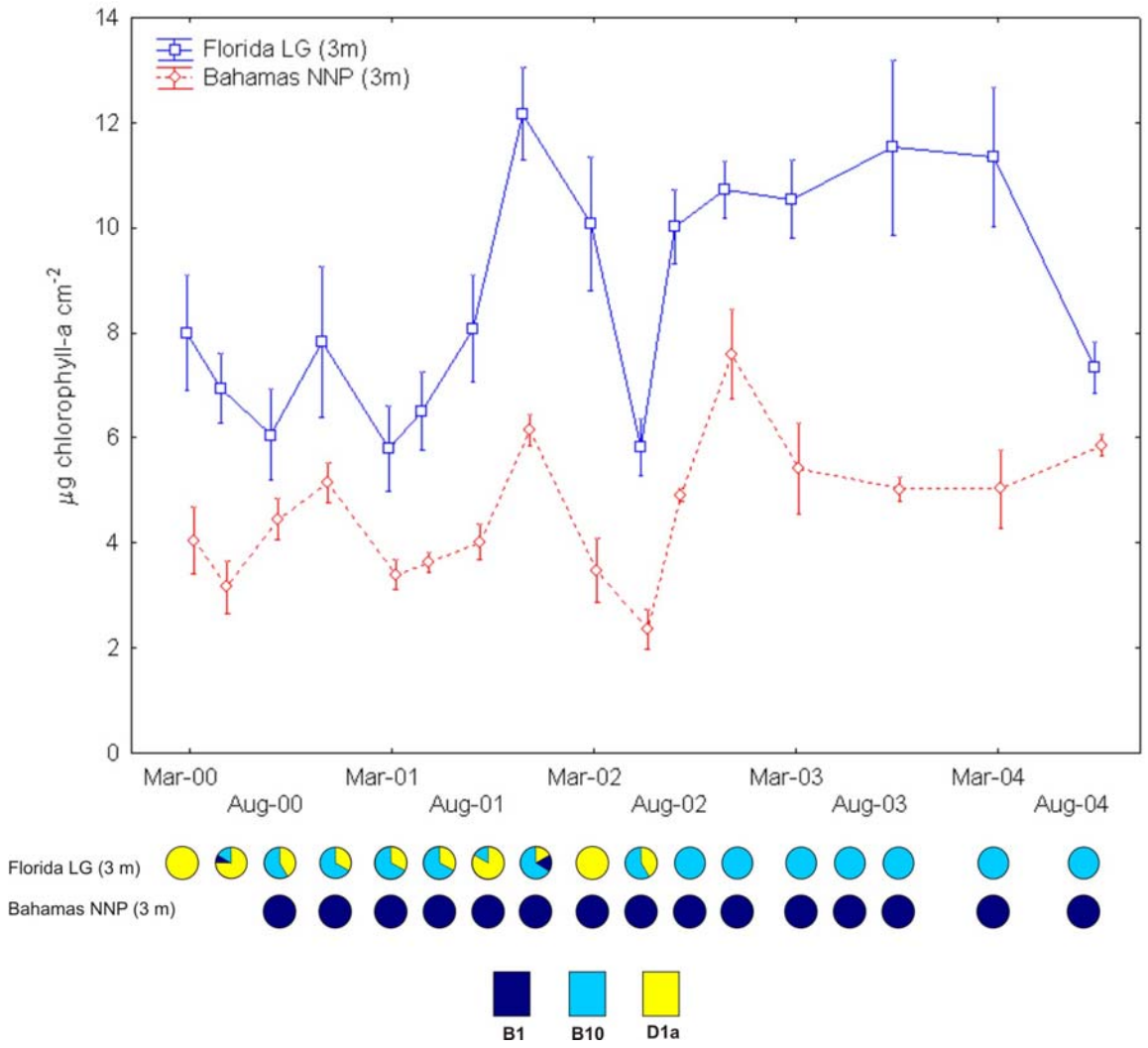


Figure 6.3: *Montastrea annularis* chlorophyll-a density in relation to time of year from two shallow (3m) reefs of the Florida Keys and Bahamas. Data are expressed as mean ± SE. *Symbiodinium* spp. type data (Fig. 5.1) is included for reference.

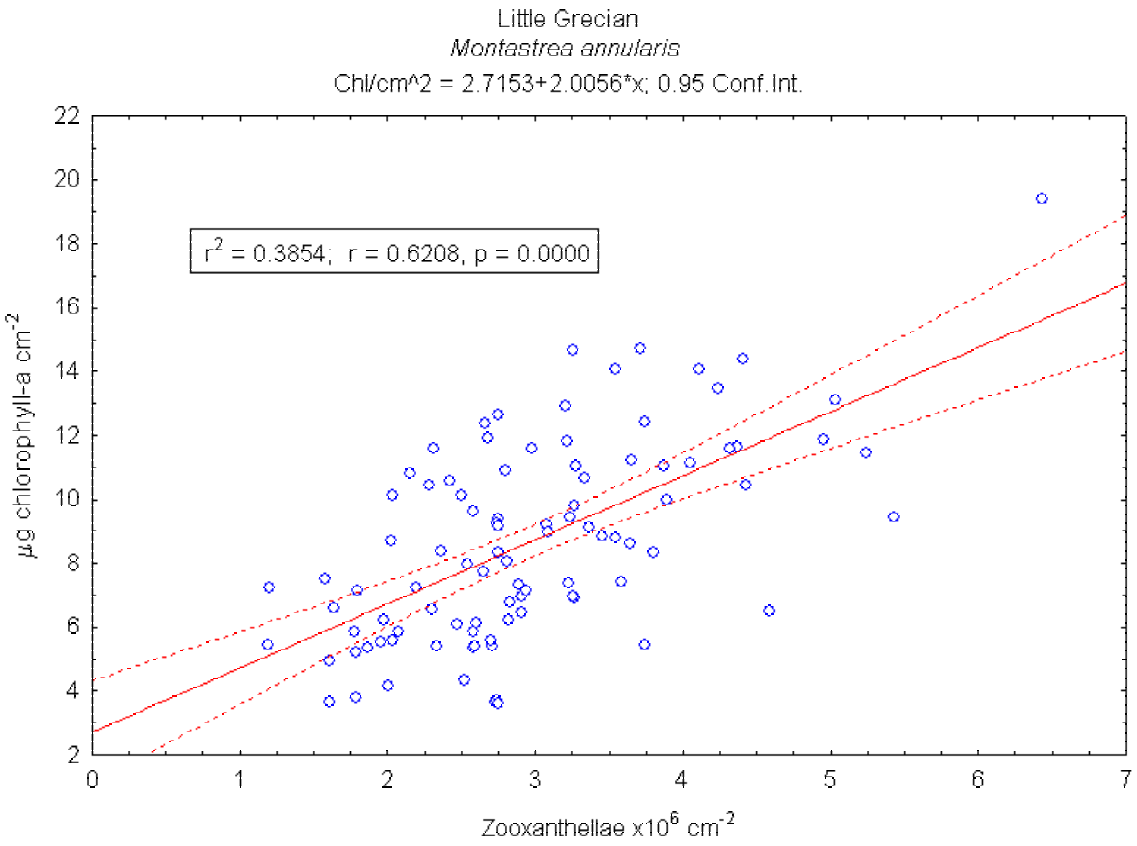


Figure 6.4: Graph of chlorophyll densities vs. zooxanthellae densities in colonies of *Montastrea annularis* from Little Grecian fore reef (3 m) in Key Largo Florida.

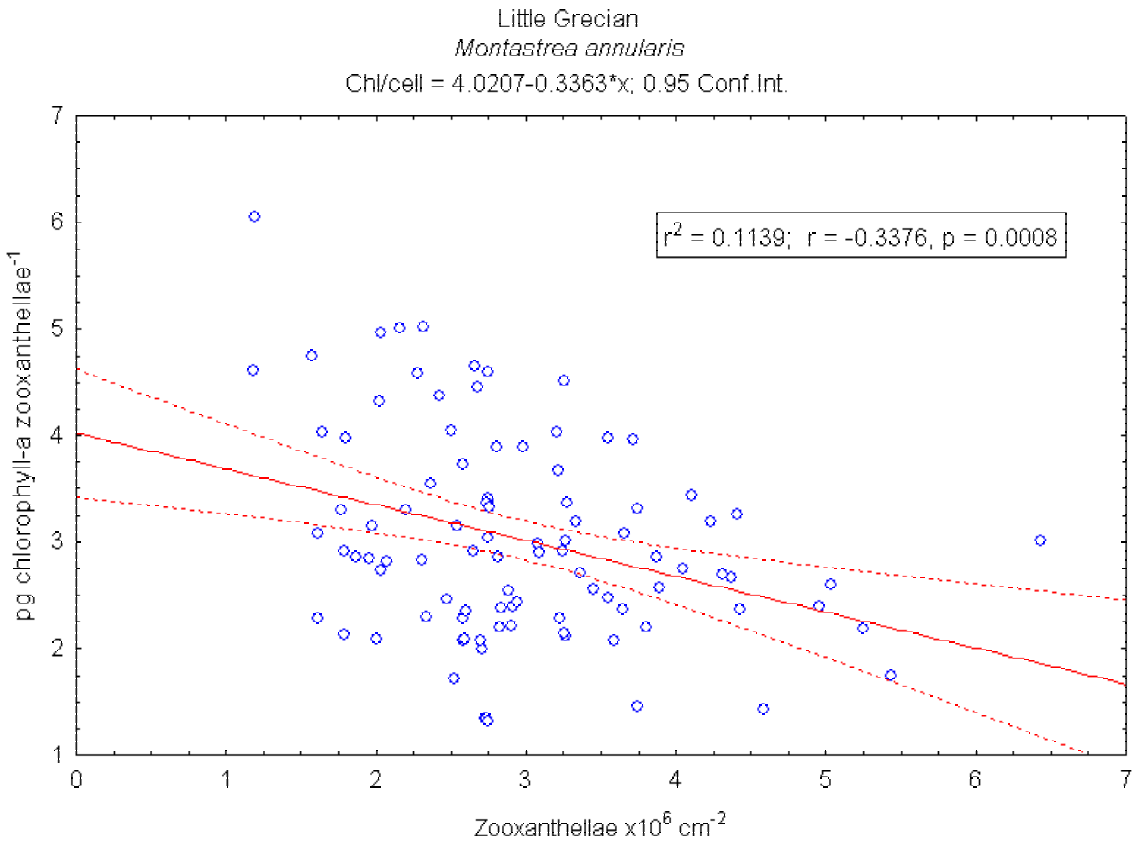


Figure 6.5: Graph of chlorophyll per cell vs. zooxanthellae densities in colonies of *Montastrea annularis* from Little Grecian fore reef (3 m) in Key Largo Florida.

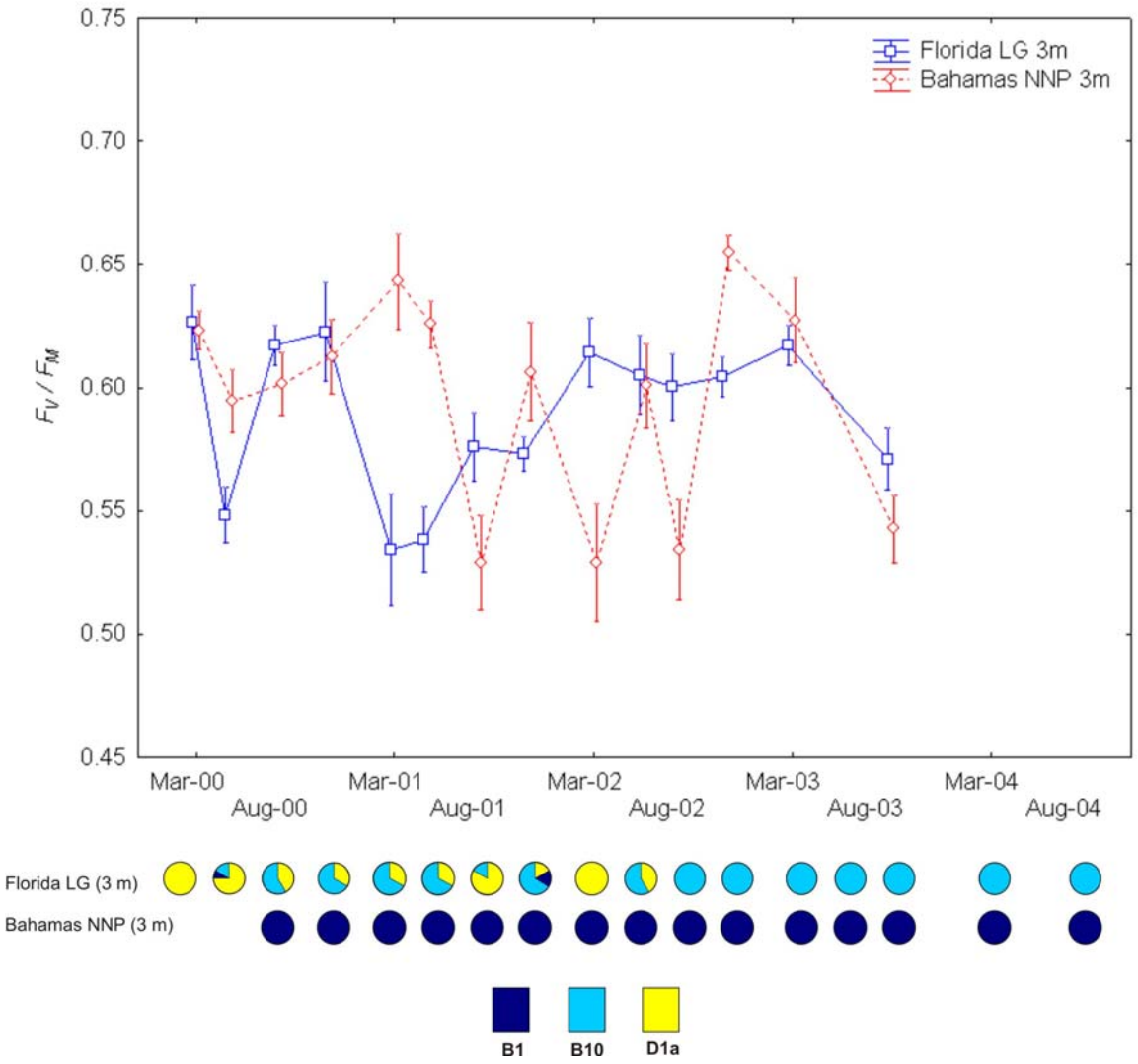


Figure 6.6: *Montastrea annularis* photosynthetic capacity of PSII in relation to time of year from two shallow (3m) reefs of the Florida Keys and Bahamas. Data are expressed as mean ± SE. *Symbiodinium* spp. type data (Fig. 5.1) is included for reference.

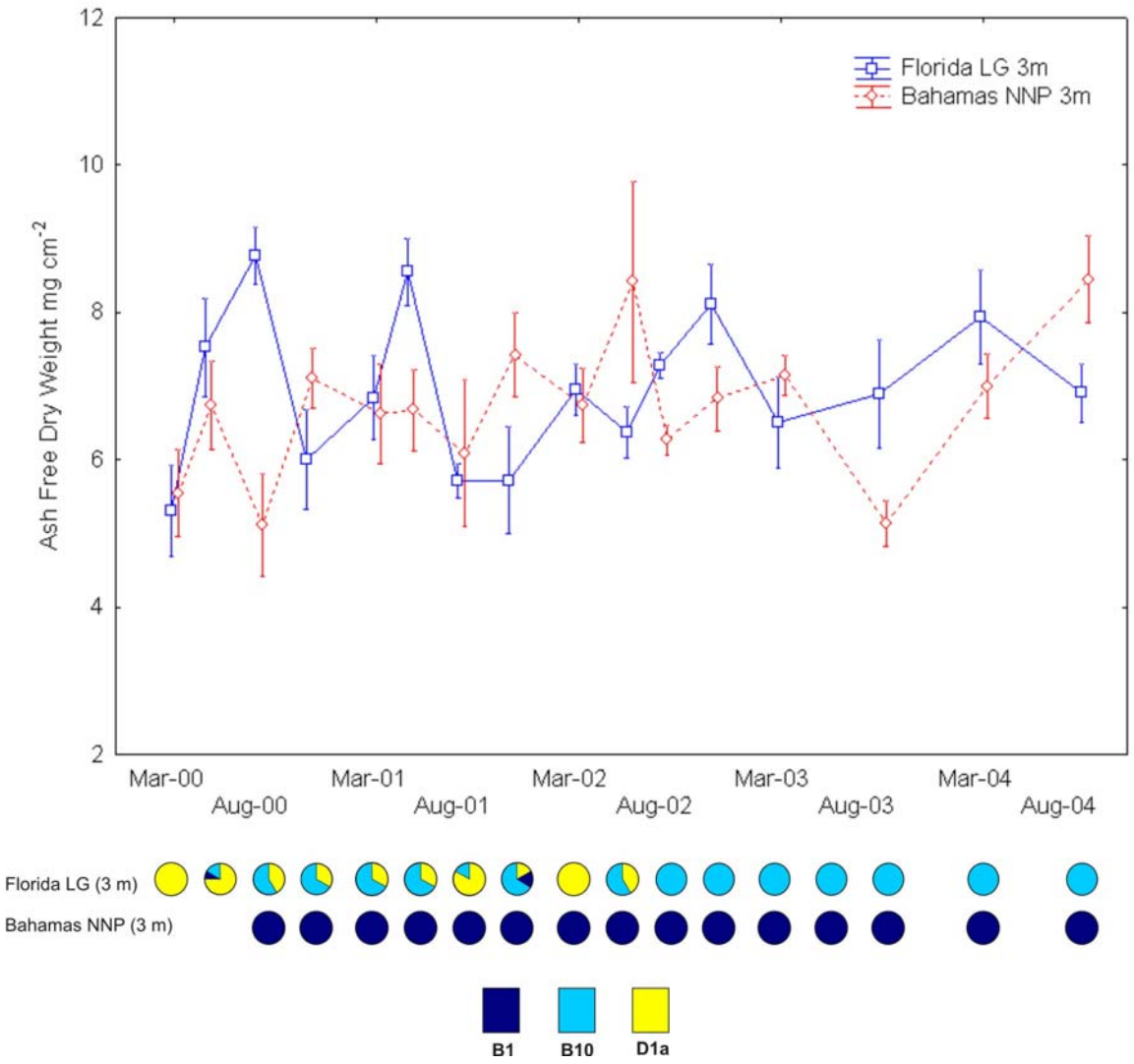


Figure 6.7: *Montastrea annularis* tissue biomass in relation to time of year from two shallow (3m) reefs of the Florida Keys and Bahamas. Data are expressed as mean ± SE. *Symbiodinium* spp. type data (Fig. 5.1) is included for reference.

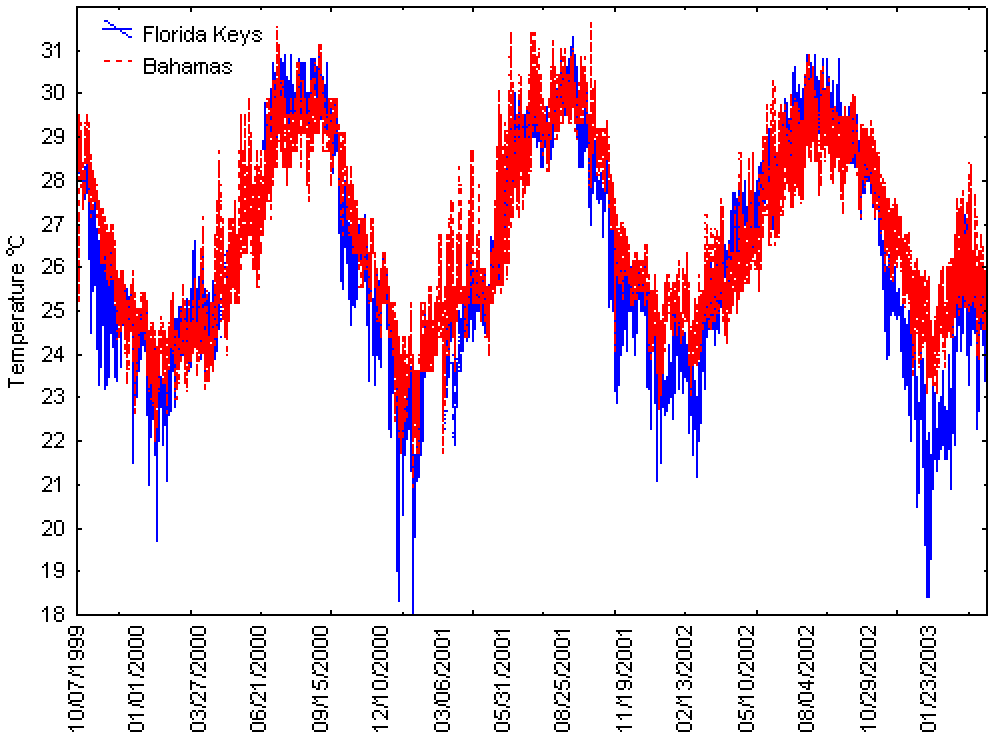


Figure 6.8: Hourly sea surface temperature from Molasses Reef in the Upper Florida Keys and North Norman's Patch Reef in the Bahamas.

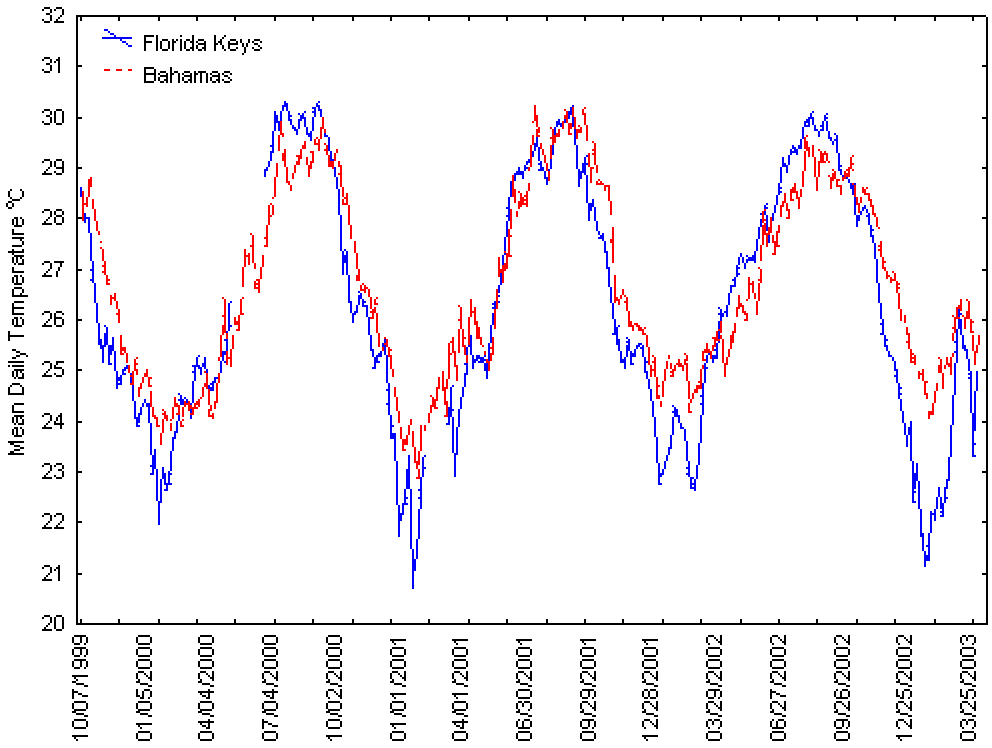


Figure 6.9: Mean daily sea surface temperature from Molasses Reef in the Upper Florida Keys and North Norman's Patch Reef in the Bahamas.

CHAPTER 7

CONCLUSION

The goal of this dissertation was to investigate patterns in cnidarian-algal symbioses, from the onset of symbiosis in larval hosts through the fidelity of adult corals for their dominant *Symbiodinium* spp. partner. I was also specifically interested in functional differences between different *Symbiodinium* spp. types and the effect that they have on the physiology of the host-symbiont association (holobiont).

Chapter 2 investigated the onset of symbiosis in a model cnidarian, the scyphistomae (polyp) stage of the jellyfish *Cassiopea xamachana*. Azooxanthellate scyphistomae were used as a means of detecting free-living *Symbiodinium* spp. from various habitats in the Upper Florida Keys. This experiment was repeated seasonally to look at whether patterns of infection varied on a seasonal basis.

Infection rates of azooxanthellate scyphistomae increased with time of exposure to the surrounding seawater. Infection rates after five-day exposures were lowest during the coldest time period sampled (March) and increased to 100% for the remaining time periods sampled (May, August, November). All four different sub-generic clades of *Symbiodinium* (A, B, C, and D) that associate with scleractinian corals were taken up by these polyps. However, the type of symbiont taken up did not correspond to types found in other nearby symbiotic hosts. Adult *C. xamachana* jellyfish from the same location were found to only harbor *Symbiodinium* sp. type

A1, suggesting that some sort of post-infection sorting must take place resulting in the displacement of other types. This pattern is similar to results observed in other symbiotic cnidarians (e.g. Coffroth et al. 2001).

Chapter 3 investigated how symbiotic associations varied in six species of broadcast spawning corals (*Acropora palmata*, *A. cervicornis*, *Siderastrea siderea*, *Montastrea faveolata*, *M. annularis* and *M. franksi*) from various habitats in the Florida Keys and Bahamas. I repetitively sampled tagged colonies and determined their dominant symbiont type beginning in August 1998 and regularly from March 2000 to August 2004.

Most colonies investigated were found to have extremely stable associations with their dominant type of *Symbiodinium*, showing either no variation or very little variation over five years of seasonal sampling. However, certain colonies of *M. annularis* and *M. franksi* from the Florida Keys underwent dynamic shifts in their symbioses, from mixed symbiont associations including the stress tolerant *Symbiodinium* type D1a to highly stable associations with types of clade B *Symbiodinium*. This change in symbiont assemblage was attributed to recovery from the 1997-1998 bleaching event. Chapter 4 provides additional information regarding intracolonial variation in *Montastrea* spp. corals, suggesting that symbiont distributions are heavily influenced by light intensity.

Chapter 5 provided additional information on how coral-algal symbioses vary through time in three species of brooding corals (*Agaricia agaricites*, *Porites astreoides*, and *Siderastrea radians*). Brooding corals are predicted to have more stable symbiotic associations because they inherit zooxanthellae from their maternal parent. All three species had extremely stable symbioses, with absolutely no variation in symbiont type over time or in relation to bleaching.

Chapter 6 investigated the influence of symbiont type on the physiology of the holobiont. Five different physiological parameters were measured seasonally from March 2000 to August 2004 from shallow *M. annularis* colonies in the Florida Keys and the Bahamas (zooxanthellae density, chlorophyll-a density, chlorophyll-a per cell, photosynthetic capacity of PSII and host tissue biomass). Colonies from Florida experienced mixed symbiont assemblages, including stress tolerant D1a, from March 2000 to May 2002 followed by stable associations with type B10 *Symbiodinium* from August 2002 to August 2004. Colonies from the Bahamas had stable symbioses with type B1 throughout this period. Statistical comparisons of *M. annularis* colonies of these two reefs revealed significant differences between when the symbiont communities were different in terms of zooxanthellae density, photosynthetic capacity and tissue biomass. When the symbiont communities stabilized, these significant differences were no longer apparent. This result suggests that symbiont type has a significant effect on the physiology of the holobiont, a central prediction of the adaptive bleaching hypothesis (ABH) (Buddemeier and Fautin 1993).

APPENDIX

A- HIGH DIVERSITY AND HOST SPECIFICITY OBSERVED AMONG SYMBIOTIC DINOFLAGELLATES IN REEF CORAL COMMUNITIES FROM HAWAII[6]

[6] LaJeunesse TC, Thornhill DJ, Cox EF, Stanton FG, Fitt WK and GW Schmidt, 2004. Coral Reefs. Reprinted with permission of the publisher.

Abstract:

The Hawaiian Islands represent one of the most geographically remote locations in the Indo-Pacific and is a refuge for rare, endemic life. The diversity of symbiotic dinoflagellates (*Symbiodinium* sp.) inhabiting zooxanthellate corals and other symbiotic cnidarians from the high islands region was surveyed. From the18 host genera examined, 20 genetically distinct symbiont types (17 from clade C, 1 of clade A, 1 of clade B, and 1 of Clade D) were identified. Most were specific to a particular host genus or species. Nearly 50% of these are new symbiont types not identified in previous surveys from Western and Eastern Pacific hosts. The absence of a dominant generalist symbiont among Hawaiian cnidarians is in marked contrast with the community structures observed among the host taxa of the Great Barrier Reef and Caribbean. Low host diversity in Hawaii, combined with atypical physical-environmental conditions and geographic isolation, appears to have enabled the formation of a coral reef community comprised of symbionts exhibiting strong host specificity. This contrasts with other reef systems that are dominated by a few prevalent generalist symbionts inhabiting a wide range of coral hosts.

Introduction:

Defining the coral-algal symbioses of Hawaii is of interest for many reasons. Encircled by the waters of the North American Countercurrent, this archipelago is among the most isolated subtropical regions in the Indo-Pacific (Simon 1987; Veron 1995). Due to its limited exchange with other central Pacific reef systems, Hawaii has the highest number of endemic corals in the Pacific (Hughes et al. 2002). Therefore Hawaii may potentially be a source of novel symbiont-host combinations.

Hawaii's high latitude and proximity to up-welled cold eastern waters has mostly spared its corals from the severe bleaching and mortality accompanying abnormally warm sea-surface temperatures associated with El Niño-southern oscillations. Such events have severely impacted shallow reef systems throughout the Indo-Pacific and Caribbean (Hoegh-Guldberg 1999), and have led in some cases to community shifts among coral taxa and their symbionts (Loya et al. 2001; Glynn et al. 2001). The Hawaiian high islands represent a relatively undisturbed region of the Pacific, prompting our investigation of coral-algal symbioses in this 'pristine' system.

Assessing the genetic diversity and phylogenetic relatedness of *Symbiodinium* spp. from reef systems around the world reveals much about the ecology and evolution of these organisms and their host relations. While host-specific and/or rare symbiont types comprise the majority of symbiont diversity, these communities are dominated by few symbionts occupying a broad range of host taxa. Different climatic and geological changes experienced by the Caribbean and Pacific over the last 3 to 4 million years have probably contributed greatly to the contrasts in relative symbiont diversity between reefs from these distinct oceanic provinces (LaJeunesse et al. 2003). Largely due to the regional success of Clade B *Symbiodinium* spp. since the Pliocene-Pleistocene transition, the number of specialized symbionts found in the Caribbean is greater, (relative to host diversity) than that which is found on a reef system from the GBR (LaJeunesse et al. 2003, unpubl.).

Existing physical environments, climate history, and geographical isolation are the strongest determinants for the distribution and diversity of most organisms. For symbionts however, taxonomic diversity and abundance of suitable host taxa (i.e. habitat diversity and availability from the symbiont point of view) is probably an additional contributor to the diversification and distribution of *Symbiodinium* spp. Analyses of ribosomal internal transcribed

spacer DNA (ITS sequences) have identified numerous host specific symbiont types that are recently evolved from a few ancestral generalists. The majority of symbionts (> 85%) consist of taxa associated with a single host genus or species. These findings emphasize the importance of niche partitioning through host specialization in driving symbiont speciation.

There are ecological and evolutionary trade-offs between symbionts associating with numerous host taxa versus specializing with a narrow host range (Futuyma and Moreno 1988). Selection pressures supporting the maintenance of a generalist or the evolution of specialists may shift depending on the abundances and diversity of host taxa (Law 1985). As consequence of Hawaii's isolation, low colonization rates combined with periodic extinctions have suppressed host diversity in the coral reef community since its replacement following the Miocene period (Kay and Palumbi 1987; Jokiel 1987).

Appraisal of the *Symbiodinium* spp. diversity in Hawaii contributes to our understanding of coral symbiosis by providing biogeographic data from a previously unsurveyed, highly endemic, sub-province of the Pacific. Moreover, baseline information gleaned from a relatively un-impacted reef system allows a test of the hypothesis that biological interactions influence the apparent inverse relation between host and symbiont diversity.

Methods:

Collections:

Host Cnidarians (multiple individuals from each taxon) were collected by SCUBA or mask and snorkel from two locations on the island of Oahu, Kaneohe bay on the south eastern side and from Haleiwa Trench, Wailua Bay on the north shore. While the Islands of Kauai, Hawaii, and Maui, among others, could not be surveyed, given that the same symbioses were

found on either end of Oahu, indicates that the symbiont assemblages from these high islands are probably similar. Some partner combinations remain constant over wide geographic distances (Loh et al. 2001). These stable patterns are also observed across large reef communities, such as the entire western Caribbean, where the same host-symbiont associations from equivalent reef habitats are maintained over wide geographic ranges (LaJeunesse et al. unpubl.). Coral fragments of 3-5 cm^2 where stripped of tissue using an air brush (Baker and Rowan 1997) and the resultant slurry of mucus, animal cell debris, and dinoflagellates was homogenized using a Tissue Tearror homogenizer (Biospec Ind.). After centrifugation at 800-1000 g, the resulting algal pellets were transferred to a 1.5 ml microcentrifuge tubes and preserved in 20% dimethylsulfoxide, 0.25M EDTA, sodium chloride-staturated water solution (Seutin et al. 1991). The symbionts from soft bodied Actinarian, Zoanthidian, and Scyphozoan taxa were isolated as previously described (LaJeunesse 2002). Porites compressa and *Montipora capitata* (=*verrucosa* Maragos 1995) displaying wide morphological variation under identical environmental conditions were sampled extensively to determine if any of these showed differences in their symbiont affiliations.

DNA extractions, PCR, DGGE, sequencing and phylogenetic analyses:

Nucleic acid extractions on 10 to 30 mg of dinoflagellate material were conducted using a modified Promega Wizard genomic DNA extraction protocol (LaJeunesse et al. 2003). The internal transcribed spacer region (ITS) 2 was amplified from each extract using primers "ITS 2 clamp" and "ITSintfor 2" (LaJeunesse and Trench 2001) with the touch-down thermal cycle given in LaJeunesse (2002). Products from these PCR reactions were electrophoresed on denaturing gradient gels (45-80%) using a CBScientific system (Del Mar, CA). As described in

detail by LaJeunesse (2002) and LaJeunesse et al. (2003), prominent bands diagnostic of each unique profile were excised, reamplified, and sequenced.

The combination of DGGE with direct sequencing provides a redundant "double check" for sequence differences attributed to intragenomic variation within the ribosomal loci and for identifying the presence of multiple symbiont types. A high proportion of ribosomal variants reported by investigators sequencing 'blindly' appears to be artifactual, probably resulting from PCR errors or the cloning and sequencing of rare variants (occurring throughout the repeat array but low in copy number) within the genome (Diekmann et al. 2003, LaJeunesse et al. unpubl.). Sequences were aligned manually using Sequence Navigator version 1.0 software (ABI, Division of Perkin Elmer, Foster City, CA). The sequence dataset of clade C comprise of types distinguished by few base substitutions and/or insertion/deletions. For this reason, maximum parsimony, which does not assume a particular model of molecular evolution and allows for incorporating informative sequence gaps designated as a 5th character state, was used for creating an unrooted clade C phylogeny using the default settings of PAUP 4.0b10 (Swofford 1993). The phylogenetic methods, maximum likelihood and neighbor-joining excluding gaps as a 5th character state, supported the same topology derived by maximum parsimony (results not shown).

Results:

A total of 20 distinctive symbiont types (17 clade C, 1 clade B, 1 clade A and 1 clade D), as distinguished by PCR-DGGE and sequencing of the ITS 2 region (LaJeunesse 2001, 2002; LaJeunesse et al. 2003) (Fig. A.1), were identified from the sampling of twenty-three host species representing 18 genera (Table A.1). Similar to recent findings on the southern GBR

(LaJeunesse et al., 2003), the community of symbiotic cnidarians maintained a disproportionate number of symbioses with Clade C types, while symbioses involving types from clades A, B, and D were rare (Fig. A.2). However, the *Symbiodinium* spp. community characterized from the Hawaiian host assemblage is atypical of the community structure observed from Caribbean and Western Pacific reefs where numerous host taxa share a common symbiont type (LaJeunesse 2002, LaJeunesse et al. 2003, unpubl.). Most Hawaiian host genera associated with symbionts not found in other host taxa. The even distribution of symbionts among various host taxa is more pronounced in Hawaii than any other reef system surveyed to date (Fig A.2).

An unrooted Clade C phylogram, derived by the method of parsimony comparing ITS 2 sequences, depicts a radiation or hard polytomy of derived symbionts from generalist ancestors, C1 and C3 (Fig. A.3), prevalent in symbioses throughout the Caribbean and GBR (LaJeunesse et al. 2003). Of potential ecological significance, C1 and C3 were found only in host species least common among those that were surveyed. Type C1f, most prevalent among Hawaiian coral taxa (Fig. A.1), is a unique type not yet found to date in western or eastern regions of the Pacific (LaJeunesse et al. 2003; Baker and LaJeunesse unpubl. data). Many other types identified (Fig. A.1) may also be endemic to this region of the Pacific. The age of Hawaii's high Islands (5.3-1.8 mya) (Claque et al. 1987) is old enough for specialized endemics to have evolved ITS 2 sequence differences from west Pacific progenitors as determined by molecular clock calculations (roughly1 base change per million years; Richardson et al. 2001; LaJeunesse submitted). Sampling from Johnston Atoll, the Line Islands and other reef systems close to Hawaii must be surveyed in order to determine if the distribution of these endemics extends to the rest of the central Pacific.

Populations of the corals *Montipora capitata*, *M. flabellata*, and *Porites compressa* were associated with two specialized symbionts whose distributions depended on host colony depth. Type C31 was found in most morphological variants of *M. capitata* surveyed above 15 meters; however, the less common shallow-dwelling orange form of *Montipora capitata* associated exclusively with type D1a in clade D (Fig. A.4a). M. *patula* colonies above 10 m also hosted D1a (cf. Rowan and Powers 1991). The symbiont described as *Symbiodinium kawagutii* from clade F (Trench and Blank 1987; LaJeunesse 2001) is most probably not the "true" symbiont of *M. capitata* (=*verrucosa*) but more likely a contaminant from the culturing process (Santos et al. 2001, LaJeunesse 2002). Type C15, observed previously in *Porites* spp. from the West Pacific and Western Indian Ocean (LaJeunesse et al. 2003, unpubl.), was also identified in each of the five *Porites* species from Hawaii. Morpho-types of *Porities compressa*, sampled below10 meters contained the endemic C15b, a symbiont subsequently found in the encrusting octocoral, *Sarcothelia* sp., living at similar depths. A purple-blue morph of *P. compressa* collected below 10 m on the Island of Hawaii and transplanted more than10 years ago to a shallow (1 m) patch reef in Kaneohe Bay, Oahu (C. Hunter pers. comm.), possessed the deep water C15b specialist (Table B.1).

Discussion:

This survey of symbiotic dinoflagellates from Hawaiian corals contributes to knowledge concerning the biology and biogeography of these symbioses. Ultimately these data will improve our understanding of the ecological and evolutionary patterns and processes exhibited by animal-algal endosymbioses. Of the 7 sub-generic clades described for the genus *Symbiodinium*, members of clade C dominate symbiotic invertebrates of the Pacific (Baker and

Rowan 1997, Pochon 2001, LaJeunesse et al. 2003). "C" types are highly prevalent in Hawaiian coral symbioses and their diversity is rich relative to low host diversity of this region. Absence of a dominant generalist combined with a high number of symbioses involving specialized symbionts, accounts for this proportionally high diversity relative to other reefs systems around the world (Fig A.5). Several factors probably contribute to the greater abundance of host specialists versus a community dominated by few generalists. 1) Hawaii has a high composition of coral taxa in the genera *Porites*, *Montipora* and *Pocillopora* that associate with specialist clade C *Symbiodinium* (Fig A.2); 2) geographic isolation has resulted in the evolution of endemic forms closely related to specialist types found in the western Pacific; 3) low host diversity in Hawaii over time possibly influenced the selection for host specialization among symbionts (Law 1985); 4) since the Miocene-Pliocene transition, paleo-climates in Hawaii may have promoted greater rates of niche diversification through variable physical-environmental conditions.

Host-symbiont specificity:

While the extent to which most corals may combine with different algal partners is unknown, emerging data indicate patterns of interaction that show varying degrees of specificity. Partner associations grade from those that appear highly specific (e.g. one to one) to those displaying greater flexibility (Baker 2003). Even among flexible partnerships, these associations display strong preference toward a certain partner combination in particular reef habitats over extensive biogeographic regions (LaJeunesse and Trench 2000; Loh et al. 2001, Coffroth et al. 2001). Foremost, the existence of a particular symbiont type in an ecosystem is dependent on the presence of a host taxon, with the physical environment being of secondary importance

(LaJeunesse 2002). These observations are explainable by evolutionary processes. The host cell environment, along with some modification by the external environment, acts as a major selective influence promoting niche diversification and speciation of symbiont taxa. Specialists that comprise the majority of symbiont diversity world-wide have evolved from ancestral generalists since the late Miocene-early Pliocene (LaJeunesse submitted), a period when, coincidently, Hawaiian reefs underwent a major faunal turnover (Kay and Palumbi 1987).

The process by which a host acquires symbionts from generation to generation has direct consequences in the diversification of specialized symbionts. In many symbiotic systems where host offspring acquire their symbionts directly from the parent (vertical transmission), genetic isolation and selection pressure promotes symbiont specialization (Douglas 1998, Loh et al 2001). Most corals in the Indo-Pacific broadcast spawn, producing offspring that must acquire symbionts from environmental sources (horizontal transmission). *Porites* and *Montipora* are common coral genera in the Indo-Pacific and are especially abundant in Hawaii. While species from these genera typically broadcast spawn, they release eggs containing vertically transmitted symbionts from their mother (Richmond and Hunter 1990). Together with the taxa from *Pocillopora*, brooders and vertical transmitters, each maintains specific associations with distinctive sub-cladal lineages within clade C (Fig. A.3).

While the process of vertical symbiont acquisition from generation to generation favors the evolution of specialist symbiont lines (Douglas 1998), high specificity and hence stability are observed in those hosts that must acquire symbionts from the environment (Wilkinson 2001). The same symbiont inhabited adults and juveniles of the mushroom coral *Fungia scutaria*, a broadcaster with horizontal symbiont transmission, sampled from Kaneohe bay and the north shore of Oahu. This 'homologous' symbiont, C1f, achieves optimal densities in Fungia scutaria

larva, whereas experimentally introduced heterologous types C15 , Cld, and C31 from *P. compressa*, *P. damicornis*, and *M. capitata* respectively, infect at lower frequencies and achieve lower densities in *F. scutaria* larvae (Weis et al. 2001). It seems that aspects of host biology, other than mode of symbiont transmission, are important in the evolution of host specialization among *Symbiodinium* spp..

Partner stability:

The endemic, numerically dominant, Hawaiian coral *Porites compressa*, displays great variation in colony morphology. It also has little preference for particular reef environments (Hunter 1988). All morpho-types surveyed above 10 meters from windward and leeward sites shared a single symbiont type, C15 (Table A.1). One exception was found in the transplanted colony now living on a shallow patch reef in Kaneohe Bay. Originally collected from a depth below 10 meters off the Island of Hawaii (C. Hunter personal comm.), it possesses a related yet ecologically distinctive symbiont, C15b. This 'type' probably constitutes a low-light specialist with a narrow host range as it was only identified from *P. compressa* and *Sarcothelia* sp. growing below 15 meters. That the symbiont population in the transplanted *P. compressa* did not undergo a shift to C15 after 10 years raises fundamental questions regarding the stability of this symbiosis. Possibly, these deep morphs of *P. compressa* have speciated, and as is the case for many cnidarian-dinoflagellate symbioses, the biochemical/genetic backdrop of this 'different' host is a greater determinant in the distribution of particular symbionts than the external environment (LaJeunesse 2002, LaJeunesse et al. 2003). Perhaps the initial change in environment did not inflict the proper stress to cause a shift from the established symbiont population to one better adapted as some have proposed (Buddemeier and Fautin 1993, Baker

2001). Physiological comparison between this transplant and nearby 'shallow' *P. compressa*, may provide a functional explanation for this host-symbiont stability.

Unusual symbioses:

Symbionts from the clade D lineage of *Symbiodinium* are rare except in host communities that experience extreme ranges in sea surface temperature or in certain hosts 'recovered' from bleaching (Baker 2001, Toller et al. 2001). Two Hawaiian montiporid corals, *M. patula* and the rudy-orange morph of *M. capitata*, associate specifically with symbiont D1a at depths above 10 m (cf. *M. patula* from Rowan and Powers1991). Discovery of these unusual symbionts in 'stable' associations may exemplify an 'adaptive shift' from a normally opportunistic generalist symbiont to one that has assumed an ecological fitness not observed in other biogeographic regions. The symbiosis of the orange *M. capitata* form with D1a (Fig. A.4a) may explain its unpalatability with certain corallivores (E. Cox unpubl.). While the evolution of new partner combinations has eco-evolutionary implications in adaptation to climate change (Buddemeier and Fautin 1993) and new habitat colonization, it may also benefit certain associations by lowering predation pressure (Augustine and Muller-Parker 1998).

Biogeography and Endemism:

Defining the diversity and phylogeny of *Symbiodinium* spp. in Hawaiian coral symbioses contributes greatly to the growing database of their biogeographic distributions, relative endemism, and host specificity over spatial dimensions. Reef systems can be characterized by the community of *Symbiodinium* spp. living in association with its host assemblage. Like their hosts, these symbiont communities consist of species with unique biogeographic distributions.

Some are found in distant provinces or even different oceans, while many are endemic specialists more localized to a particular reef system. Regionally distinctive types provide new markers for defining distinctive biogeographic provinces or sub-provinces of genetic connectivity, even over limited spatial scales such as the Caribbean (LaJeunesse et al. in prep.).

The planula brooding pocilloporids host several specific symbionts (Fig A.3). Type C1c has an extensive geographic distribution and is found in *Pocillopora* spp. from the GBR, Okinawa, and the eastern Pacific (LaJeunesse et al. 2003; unpubl.). On *Pocillopora* spp. dominated reefs of the eastern Pacific (Porter 1974), type C1c also occurs in the coral genus *Pavona* and an unidentified actiniarian species (Baker and LaJeunesse unpubl.). The numerical dominance of *Pocillopora* spp. in this region of the Pacific may explain, in part, the shift of this abundant symbiont type to other host taxa. The other 'pocilliporid' *Symbiodinium* sp. surveyed from Hawaii may have more limited biogeographic distributions. This includes type C1d, which also occurs in some *P. damicornis* from the eastern Pacific but, has yet to be found in the western Pacific. Finally, types C1g and C34 appear to be part an even more narrowly distributed symbiont assemblage, possibly endemic to Hawaii (Fig A.3).

The distribution of a particular symbiont may provide an additional tool in defining/assessing long-range genetic connectivity between coral reefs. The specialized symbiont C31 hosted by *M. capitata* in Hawaii is also common in montiporids from reefs around Okinawa (unpubl.). *Montipora* spp. from the GBR host different types that belong to the same 'montiporid' *Symbiodinium* sub-clade of clade C. Further surveys conducted on island archipelagos closest to Hawaii will be necessary to determine the exact proportion of true endemic types to these islands. Regardless, the survey in Hawaii indicates that the Central

Pacific province contains many symbiont types different from those found in hosts from the GBR (LaJeunesse et al. 2003, unpubl.) and Okinawa (unpubl.).

Table A.1 Host species, symbiont type and collection depth (meters) of samples from Oahu Hawaii, north central Pacific. Numerals in parentheses indicate the number of colonies independently sampled.

HOST	DEPTH (meters)	Symbiont type (Genbank accession)	
Scleractinia			
Acroporidae			
Montipora capitata (orange color morph)(4)	1.0-2.0	D1a	
Montipora capitata (various colony morphologies) (14)	1.0-5.0	C31	(AY258496)
Montipora capitata (deep encrusting form) (2)	20.0	C35a	(AY258500)
Montipora flabellata			
	2.0	C32	(AY258498)
Montipora flabellata			
	10.0-20.0	C32a	
Montipora patula (2)	3.0-15.0	D1a	
Montipora patula			
	20.0	C31	
Pocilloporidae			
Pocillopora damicornis (dark morph) (4)	1.0-2.0	C1d	(AY258488)
Pocillopora meandrina (4)	2.0-20.0	C1c	
Pocillopora molokensis (5)	18.0-25.0	C34	(AY258499)
Pocillopora eydouxi (2)	20.0	C1c	
Pocillopora ligulata			
	20.0	C1g	
Poritidae			
Porites compressa (various colony morphologies) (19)	1.0-4.0	C15	
Porites compressa (3)	15.0-25.0	C15b	(AY258491)
Porites compressa (10 year transplant)	1.0 (>10.0)	C15b	
Porites evermanni (4)	5.0-20.0	C15	
Porites lobata (5)	2.0-20.0	C15	
Porites rus (3)	10.0-15.0	C15c	(AY258492)
Porites brighami (2)	20.0	C15	
Siderastreidae			
Coscinaraea wellsi *(1)*			
	10.0-20.0	C1	
Psammocora spp. (4)	2.0-5.0	C1f	(AY258490)
Fungiidae			
Fungia scutaria (5)	2.0-10.0	C1f	

Fungia scutaria (2) (acanthicauli)	3.0	C1f	
Cycloseris vaughani *(1)*			
	20.0	C1	
Agariciidae			
Pavona varians (7)	2.0-20.0	C27	
Pavona duerdeni (4)	10.0-25.0	C27	
Leptoseris incrustans (2)	10.0-20.0	C1	
Faviidae			
Leptastrea purpurea (6)	10.0-20.0	C1f	
Leptastrea bottae (3)	2.0	C1f	
Cyphastrea ocellina (3)	2.0	C1f	
Actiniaria			
Aiptasia pulchella (2)	1.0	B1	
Boloceroides mcmurrichi			
	2.0	C3	
Zoanthidea			
Palythoa sp. (2)	2.0-15.0	C1	
Palythoa sp.	20.0	C3	
Protopalythoa sp. (3)	1.0	C33	(AY258498)
Zoanthus pacificus (3)	1.0	C29	(AY258494)
Alcyonacea			
Sarcothelia (*edmondsoni*) sp. (2)	10.0-20.0 m	C15a	
Scyphozoa			
Cassiopeia sp.	3.0	A3	

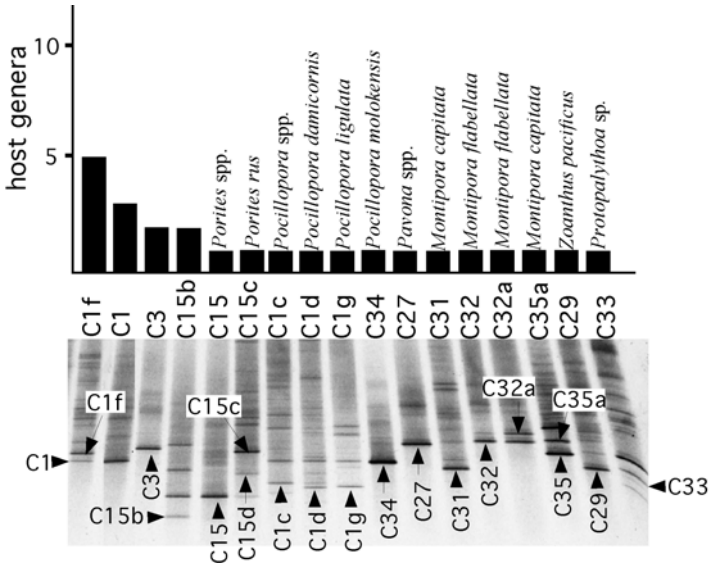


Figure A.1 The community of *Symbiodinium* spp. found among Hawaiian corals comprises primarily of types originating from clade C. While several "C" types associated with multiple host taxa, most were specific to a single host genus or species. PCR-DGGE analyses identified 17 different C type signatures whose ribosomal gene arrays were either dominated by a single sequence or exhibited two or more co-dominant variants. In patterns with multiple bands, the relative banding intensities did not differ from sample to sample, indicative of fixed intragenomic variation. Mixed populations consisting of different symbiont types within a host colony were never observed.

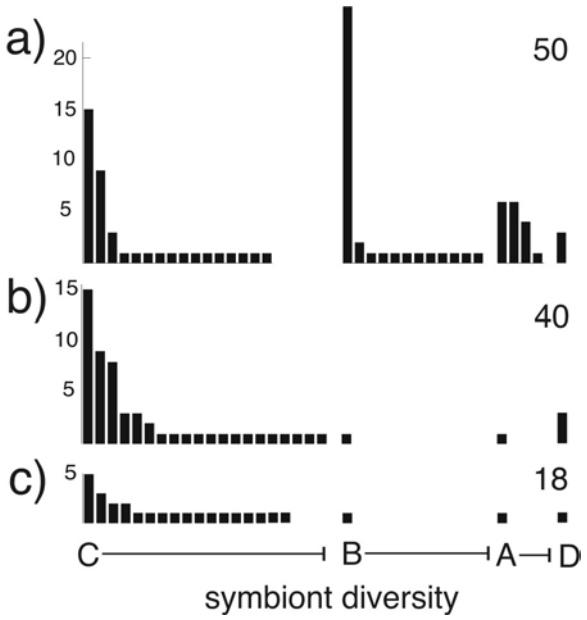


Figure A.2 Symbiont community structures and prevalence among host genera for a) the Caribbean, b) the southern GBR (LaJeunesse et al. 2003) and c) Hawaii. Hawaii lacks a host generalist as common as those found in other reef systems. This may in part, explain why Hawaii's symbiont diversity is nearly equivalent to the southern GBR with only half the number of genera and one third the number of species surveyed. Number of host genera surveyed from each region is given in the upper right hand corner of each panel.

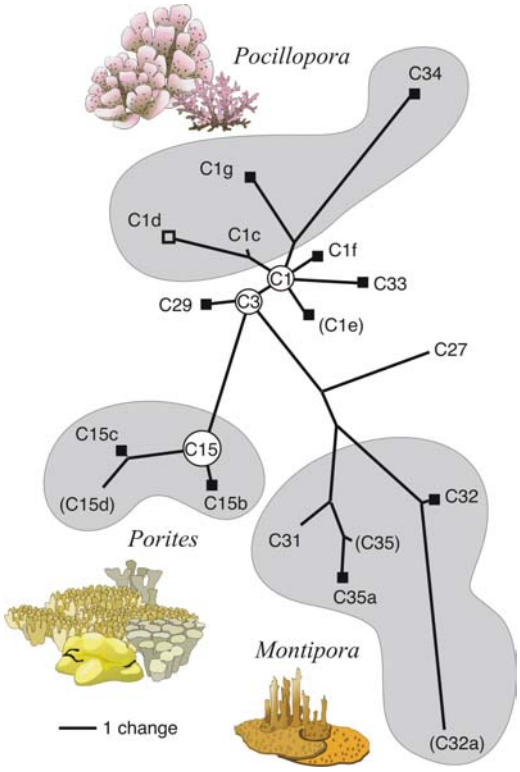


Figure A.3 Unrooted ITS 2 sequence phylogeny of clade C.*Symbiodinium* spp. based on maximum parsimony. The majority of symbiont diversity comprises host specialized forms radiated from generalist ancestors. Types C1 and C3 are ancestral generalists, and while not dominant in Hawaii, are exceptionally common among hosts examined from the western Pacific and Caribbean. The coral genera whose offspring acquire symbionts through vertical transmit ion, *Porites*, *Montipora*, and *Pocillopora*, associate with *Symbiodinium* spp. from discrete sub-clades. Solid squares indicate symbiont taxa recorded only from Hawaii. Type C1d occurs in *P. damicornis* from eastern Pacific (open square), and not identified from the west Pacific. All remaining types are found in hosts from the western Pacific.

a

b

Figure A.4 The endemic coral *Montipora capitata* (=*verrucosa*), while morphologically plastic, also has two color morphs. The ruddy-orange variety (a) associates with a *Symbiodinium* sp. from Clade D (type D1a) and is less palatable than the common "brown" variety (b). The common brown form associates with C31 above 15 meters, but hosts type C35a at greater depths.

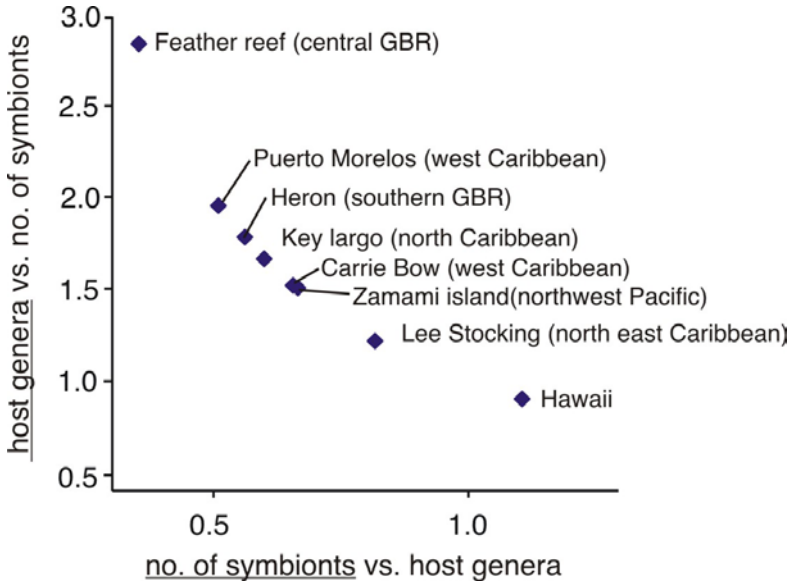


Figure A.5 The inverse ratios between host generic diversity and symbiont diversity (ITS 2 types) in reef systems from the Pacific and Caribbean. Hawaii possesses the lowest host diversity, yet exhibits a relatively high proportion of symbiont diversity. The host assemblage at Feather reef (Central GBR, unpubl.), with the most number of taxa surveyed (50 genera), contains a low number of symbionts in proportion to its host diversity. Position along this curve is probably influenced by other factors including but not limited to, historical climates, recent bleaching history, and the relative eveness of host species abundances through time.

APPENDIX

B – RESPONSE OF HOLOSYMBIONT PIGMENTS FROM THE SCLERACTINIAN CORAL *MONTIPORA MONASTERIATA* TO SHORT TERM HEAT STRESS[7]

[7] Dove S, Ortiz JC, Enríquez S, Fine M, Fisher P, Iglesias-Prieto R, Thornhill DJ, and O Hoegh-Guldberg. In submission to Limnology and Oceanography.

Abstract

Heating the scleractinian coral, *Montipora monasteriata* (Forskăl 1775) to 32^{o}C under less than 650 μmol quanta m^{-2} s^{-1}, led to bleaching in the form of a reduction in Peridinin, xanthophyll pool, Chlorophyll c_2 and Chlorophyll *a*, but areal dinoflagellates densities did not decline. Associated with this bleaching, chlorophyll (Chl) allomerisation and dinoflagellate xanthophyll cycling increased. Chl allomerisation is believed to result from the interaction of Chl with singlet oxygen ($^1O_2$) or other reactive oxygen species. Thermally induced increases in Chl allomerisation are consistent with others studies that have demonstrated that thermal stress generates reactive oxygen species in symbiotic dinoflagellates. Xanthophyll cycling requires the establishment of a pH gradient across the thylakoid membrane. Our results are therefore not compatible with a recent non-photoinhibition model of thermal bleaching that is based on an immediate thermal meltdown of the thylakoid membrane. Different morphs of *M. monasteriata* responded differently to the heat stress applied: heavily pigmented coral hosts taken from a high light environment showed significant reductions in GFP-like homologues whereas non-host pigmented high light morphs experienced significant reduction in water soluble protein content. Paradoxically, the more shade acclimated cave morph were, based on Chl fluorescence data, less thermally stressed than either of the high light morphs.

Introduction

Mass coral bleaching events witnessed over the last 20 years are characterised by drastic losses of dinoflagellate pigmentation from scleractinian corals in response to anomalous increases in sea surface temperature (Hoegh-Guldberg 1999). A statement similar to this occurs in almost every manuscript written about the effect of thermal stress on coral bleaching. As yet,

however no publication has investigated the effect of heat on the individual pigments (inclusive of carotenoids) that give these endosymbiotic dinoflagellates their characteristic coloration. The effect of these bleaching events on host or coral synthesized pigmentation is even less documented extending merely to anecdotal statements commenting on the brilliant blues, pinks and greens observed in some corals at recently bleached sites.

The aim of this study was to investigate the effect a short-term heat stress (6 h at 32 ^{o}C at a relatively low light intensity) has on all the individual pigments that give rise to coloration in the scleractinian coral *Montipora monasteriata*. At Heron Island on the Great Barrier Reef Australia, *M. monasteriata* is found at a depth of 3 to 5 m in a variety of external light environments ranging from out in the open (< 1000 μmol quanta m^{-2} s^{-1}) to the shaded mouths of caves (< 600 μmol quanta m^{-2} s^{-1}). In the open, *M. monasteriata* occurs as a range of color morphs inclusive of a purple and a brown morph. In caves, *M. monasteriata* takes on a brown or red-brown coloration (Anthony and Hoegh-Guldberg 2003). The experiment was conducted under thick cloud and provided a light regime very similar to that reported for cave *M. montipora* by Anthony and Hoegh-Guldberg (2003). The open morphs were therefore subjected to heat under lower than typical daily photon flux densities (PDF), with the cave morph being subjected to heat under a typical PFD profile. The centre question of the study was does a close inspection of all the pigments involved provide any clues as to how does heat stress rapidly develops into chronic photoinhibition.

Seasonally corals bleach and lose up to 50 % of their areal-dinoflagellate densities (Warner et al. 2002). This response appears to co-occur with reductions in host tissue dry weight and/ or host protein concentration and has been attributed to a reduction in dinoflagellates carrying capacity by the host (Fitt et al. 2002), seasonal shifts in light, temperature (Fitt et. al.

2000, Warner et al. 2002) and even nutrient availability (Fagonee et al. 1999). Corals however tend to bleach in response to changes in PFD (achieved experimental or as a result of transplantation) by reducing dinoflagellate chlorophyll (Chl) concentration as opposed to altering areal dinoflagellate densities (Falkowski and Dubinsky 1981; Hoegh-Guldberg & Smith 1989; Fitt and Cook 2001). This loss of pigmentation is usually attributed to an overhaul to the structure of the dinoflagellate light harvesting complexes analogous to the photo-acclimation observed in higher plants as they gently photo-acclimate to a change in photon flux (*aka*, photo-adaptation, Iglesias-Prieto and Trench 1997). In scleractinian corals, the overhaul of the light harvesting complexes and ensuing optimization of photosynthesis may be achieved after only a 5 or 6 day exposure to an altered PFD (Anthony and Hoegh-Guldberg 2003). Alternatively, it may take more than a month and show a differential rate of response to increasing and decreasing PFD (Falkowski and Dubinsky 1981). Restructuring the light harvesting antennae involves adjusting and repositioning the relative abundance of carotenoids (that are able to dissipate energy to heat) and chlorophylls (that predominantly harvest energy) by the differential synthesis of template proteins (Horton et al 1996).

Algae and plants that are deficient in carotenoids (eg. mutated to disable carotenoid biosynthesis) undergo rapid photobleaching when they experience increases in PFD that can lead to plant mortality (Nigoyi 1999). This rapid photobleaching is typically attributed to an excessive build up of singlet oxygen ($^1O_2$) within the light harvesting antennae due to the extended life of singlet excited state chlorophyll (^{1}Chl) that are unable to immediately dissipate energy to similarly energized neighbours (Telfer et al. 1994; Vicenti et al. 1995). Bleaching in this case is thought to follow from the self destruction of the antennae as $^1O_2$ interacts with the histidine ligands that bind chlorophyll to protein, allowing proteolysis of apoproteins (Adamski

et al. 1993; Thomas 1997). The interaction of Chl with $^1O_2$ results in the formation of chlorophyll allomers that can be chromatographically quantitated (Hynninen 1991; Wang et al. 2000; Zapata et al. 2000).

An increase in PDF results not only in an increase in xanthophyll cycling pigments in particular and carotenoids in general (Montané et al. 1998); but also increases the conversion rate of this pool to de-epoxidated forms zeaxanthin in higher plants and algae (Hagar and Stansky 1970) and diatoxanthin (Dt) in dinoflagellates (Ambasari et al. 1997, Brown et al. 1999). A conversion that is controlled by ΔpH across the thylakoid membrane generated as a result of a photochemical flow of electrons between the donor site of PSII to the acceptor site of PSI supported by either direct reduction of O_2 in the Mehler pathway (Neubauer and Yamamoto 1992) or by cyclic electron flow through PSI (Munekage et al. 2004). These and other membrane bound carotenoids perform essential functions quenching ^{1}Chl, ^{3}Chl and $^1O_2$. They have also been found to inhibit to some degree lipid peroxidation (Harvaux and Nigoyi 1999) and stabilize membranes (Tardy and Havaux 1997). Although these later roles are more usually associated with α-tocopherols (vitamin E) that are not only able to move freely within the membrane, are also able to scavenge O_2- and OH in addition to $^1O_2$ (Harvaux and Nigoyi 1999; Nigoyi 1999).

A variety of mechanisms for bleaching in response to thermal stress have been offered. At relatively elevated temperatures (32 - 34 ^{o}C), host cells appear to detach from the mesoglea, leading not only to a loss of the endodermal cell but also of the resident dinoflagellates (Gates et al.1992, Dunn et al. 2002). This phenomenon may be an illustration of the general susceptibility of Cnidarian endodermal cells to elevated temperature (≥ 32 ^{o}C, but variable for different organisms and life stages) observed by Schmid et al. (1981). Bleaching at lower temperatures (30 - 34 ^{o}C) has predominantly been attributed to the expulsion of the photosynthetically

damaged dinoflagellates from intact host cells (Iglesias-Prieto et al. 1992; Fitt et al. 2001). The susceptibility of photosynthesis to thermal stress explains the compounding effect of light on bleaching observed by many researchers (Iglesias-Prieto 1997; Jones et al. 1998). Aspects of the molecular mechanisms that result in increased photosynthetic inhibition and damage to the resident photoautotrophs is a subject of much debate. Some of these explanations place the initial target of thermal stress firmly within the dinoflagellate. These explanations are assumed in the literature by proponents of the Adaptive Bleaching Hypothesis (ABH) who argue that corals will become more resistant to thermal bleaching as they exchange thermally sensitive dinoflagellates for thermally tolerant dinoflagellates (Baker 2004). In line with this suggestion are studies that support the notions: that bleaching is initiated with a thermal disruption to dinoflagellate thylakoid membrane integrity (Iglesias-Prieto et al. 1992; Tchernov et al. 2004); or that the primary site of thermal damage is located around PSII (Iglesias-Prieto 1997; Warner et al. 1999); or that damage to PSII is secondary to damage that occurs downstream within the dark reactions of photosynthesis (Jones et al. 1998). A minority of researchers are open to the suggestion that the primary target may lie outside the photosynthesizing endosymbiont, either associated with necrotic host cells limiting the supply of key photosynthetic cofactors (eg., Ca^{2+}; Bumann and Oesterhelt 1995), or substrates (eg., CO_2; Dunn et al. 2002); or heat associated disruptions to host pigment leading to an elevation in symbiont illumination and/or a loss of any photoprotective functions provided by these host pigments (Dove 2004).

A detailed analysis of the fate of holosymbiont pigmentation after a short term heat stress can assist in sorting amongst these diverse mechanisms presented in the literature. Bleaching resulting from a loss of Chl, but not from a loss of dinoflagellate cells suggests that it is not, at least initially, driven by a loss of host endodermal cells. The existence of a proportionate

increase in the de-epoxidated form of xanthophyll despite significant visual paling would suggest that bleaching did not follow from a total loss of membrane integrity. Based on an analogy with unicellular algae, thermal effects that results in an increase in excitation pressure at PSII should seemingly be accompanied by an increase, rather than a decrease, in the xanthophylls pool. A significant increase in Chl allomerisation is suggestive of photo-oxidation by the interaction of Chl with $^1O_2$ and other reactive oxygen species. Finally, we can ask about the stability of host pigments under thermal stress.

Methods

Collection of samples. Fragments from *Montipora monasteriata* colonies were collected from the spur-and-groove reef environment (Wistari Reef, GBR Australia) described by Anthony and Hoegh-Guldberg (2003). In April 2003, purple-HL (purple) and brown-HL (brown) morphs were collected from open habitats that were free from shading by the walls of the grooves (defined as *open habitats* by Anthony and Hoegh-Guldberg, 2003); brown-LL (cave) morphs were collected from under the edge of the shelf like overhangs formed by the groove walls (defined as *overhangs* by Anthony and Hoegh-Guldberg 2003). Three fragments from distinct colonies were collected from each morph. Fragments were cut into 2 cm x 1 cm rectangles and left to recover in sheltered aquaria under running sea water for 3 days.

Experimental design. The experiment was undertaken in the open at Heron Research Station (Southern GBR, 23°33'S, 151°54'E; a 10 min boat trip from the site at which specimens were collected) during a week of dense cloud in the tanks described by Dove (2004). Light levels were monitored with an ODYSSEY PAR cosine recorder (DATAFLOW Systems Pty Ltd, New

Zealand) and did not exceed (630 μmol quanta m^{-2} s^{-1}; 1000 – 1600 h, mean = 60 μmol quanta m^{-2} s^{-1}). Equal numbers of fragments from each morph were randomly assigned to either a control or a heated temperature treatment. The water heater was turned on at 0700 h the following day, attained 32°C by 1000 h and held at that temperature until 1600 h. Chlorophyll fluorescence measurements were taken 2 h after sunset. Five fragments from each morph and treatment were frozen -20°C for subsequent dinoflagellate density, protein content and dinoflagellate sub-clade analyses. Three fragments from each morph were divided into 3 equal portions, and placed in liquid nitrogen followed by storage at -70°C in preparation for pigment analysis.

Chlorophyll fluorescence measurements. Measurements were taken using a DIVING-PAM (Pulse Amplitude Modulated) underwater fluorometer (WALZ, Germany) fitted with a blue-LED measuring light.

Determination of surface area, total soluble protein concentrations and dinoflagellate densities. The surface area was calculated using the software MATROX Inspector 2.1 (build 17, Canada) from a digital photograph of the sample.

Samples were waterpiked with seawater and homogenized, prior to centrifugation at 4000 g for 5 min. The supernatant was transferred to a clean tube and absorbance values were determined at 280 and 235 nm in a SHIMATZU UV 2450 spectrophotometer. Protein concentration was estimated using the equation proposed by Whitaker and Granum (1980).

The number of dinoflagellates was estimated by counting 6 independent sub-samples of the dinoflagellate pellet resuspended in sea-water. Samples were diluted or concentrated to

maintain between 20 and 60 cells in the field of view. A digital camera (model 3.2.0, DIAGNOSTIC Inc. U.S.A.) attached to a fluorescent microscope (model BX 41, OLYMPUS Japan) was use to take photograph sub-samples. The software MATROX Inspector 2.1 (build 17) was used to count the number of cells present in each sub-sample. The count excludes dead cells (A. Lawton, pers. comm.).

Analysis of pigmentation.

Dinoflagellate (and endolithic) pigment extraction: Frozen whole coral samples (stored at -70^{o}C) were crushed in 90 % methanol using a mortar and pestle. The solution was sonicated in iced water for 5 min, then centrifuged at 4000 g for 3 min. Supernatant was collected and transferred to a clean tube. This protocol was repeated three times to ensure a complete extraction. 0.5 ml alloquots were immediately placed in a vacuum dryer at 25°C and stored at -70^{o}C.

Preparation of standards: Diadinoxanthin (from Bacillariophyceae), Peridinin (from Dinophyceae), Diatoxanthin (from Bacillariophyceae), ß-carotene (from Cyanophyceae), Chlorophyll (Chl) c_2 (from Cryptophyceae), Chl *a* (from Cyanophyceae) and Fucoxanthin (from Bacillariophyceae) were obtained from DHI WATER AND ENVIRONMENT (Denmark). Standards were resuspended in 90% methanol and placed immediately in a vacuum dryer at 25°C, prior to storage at -70^{o}C.

Dinoflagellate (and endolithic) pigment separation: Pigments were separated with a SHIMADZU (Japan) SCL-10 HPLC linked to a SCHIMADZU SPD-M10A photodiode array

detector using the column and method proposed by Zapata et al. (2000). Pigment separation was achieved using solution A (methanol:acetonitrile:aquose pyridine) and solution B1 (methanol:acetonitrile:acetone solution) options (Zapata et al. 2000). Dried samples and standards were resuspended in 60% methanol with 0.2 ml of water added to 1 ml of the resuspended sample immediately prior to injection (Zapata et al. 2000).

Extraction and separation of (host) water-soluble pigments: Samples were air-brushed in 5 ml of 0.06 mol L^{-1} Potassium Phosphate pH 6.65; centrifuged at 4000 g for 5 min to remove dinoflagellate pellet. Protein concentration of the supernatant was determined as before using the method of Whitaker and Granun (1980). Pigments were separated by gel filtration chromatography as described by Dove (2004) on the SHIMADZU system described above.

Dinoflagellate identification. Dinoflagellate DNA was prepared and typed by denaturing-gradient gel electrophoresis (DGGE) using the methods described in LaJeunesse et al. (2003). Profiles of the internal transcribed spacer 2 (ITS2) region of nuclear ribosomal RNA genes obtained from specimens were compared to the profiles of standards generated by T. LaJeunesse (University of Georgia, U.S.A.).

Statistical analysis of data. Data were analysed in STATISTICA 6.0 (Statsoft Inc, U.S.A.) using univariate and multivariate 2 factorial analysis of variance (ANOVA). Assumptions were tested using the Cochran C test. The Student Newman-Keuls (SNK) test was used for *post hoc* comparisons of group means. In general, we have adopted the protocol of reporting summary statistics for ANOVA with significant main effects in the figures, with SNK statistics reported in

the text. Summary statistics for ANOVA with non-significant main effects are reported in the text.

Results

Montipora monasteriata held at 32°C for 6 h showed no reductions in dinoflagellate densities with the application of heat and no significant differences in dinoflagellate densities between morphs (Morph: $F_{2, 24} = 2.97$ $p = 0.07$; temperature $F_{1, 24} = 0.42$ $p = 0.52$; Fig. B.1C). Total animal soluble protein in brown-HL controls only was significantly reduced by heating (SNK, $p = 0.000$; Fig. B.1A). The relatively high protein content in brown-HL controls drove the significant interaction observed between temperature and morph (Fig. B.1A). Dark adapted Fv/Fm gave a significant effect for temperature with all morphs from the heated treatment experiencing significant chronic photoinhibition (SNK: control $>_{0.0003}$ heated; Fig. B.1D). There was also a significant interaction between morphs and temperature with the cave (brown-LL) morph apparently less photoinhibited than the open dwelling brown and purple morphs (SNK: cave heated $>_{0.0003}$ brown-HL heated = purple-HL headed, Fig. B.1D). Significantly, the cave-dwelling morph was dominated by a different dinoflagellate subclade, C21 to that of the open-dwelling brown and purple morphs. The open-dwelling brown-HL and purple-HL morphs were dominated by dinoflagellate subclade C17 (Fig. B.1B). Short-term heating lead to a visual change in the coloration of all morphs (data not shown).

The open-dwelling purple, open-dwelling brown and cave-dwelling brown morphs were distinguishable by distinct gel filtration chromatogram profiles of host homogenates determined at 420 nm and 580 nm. Chromatograms were normalized to total protein to investigate whether loss of host pigmentation attributable to GFP-homologues was occurring at a greater rate than

that of observed total protein reductions. Fractions containing pigments absorbing at 420 nm and 580 nm eluted in the void volume, at 25.6 min, 26.6 min, 27.7 min, and 31.1 min. The 25.6, and 26.6 fractions correspond to distinct oligomeric states of GFP-homologues (Dove et al. 2001). Spectra at each elution time point as determined by photodiode array match the profile of pocilloporins (Dove et al. 1995, Dove et al. 2002). The 27.7 min peak is spectrally identical to peridinin-chl a-protein (PCP; http//www.prozyme.com/technical/spectra/percp.html). In the purple-HL morph, this PCP peak is contaminated by the tail-end of the dominant pocilloporin peak and absorbs maximally in the red at 670.6 nm. In the brown-LL morph this maxima occurs at 671.7 nm (Fig. B.2).

Thirty-two degrees celsius for 6 h lead to a decreased in pocilloporin absorbance (measured as area under 580 nm chromatogram between 20 min and 35 min) per mg protein for purple-HL morphs (Table B.1) and also included a shift towards smaller oligomeric forms of the protein (from 25. 6 min to 26.6 min; Fig. B.2). Elevated temperature also led to a decrease in PCP absorbance (measured as area under 420 nm chromatogram between 20 min and 35 min) per mg protein for the cave morph (Table B.1). Purple-HL controls contained more pocilloporin than brown-LL or brown-HL morphs (Table B.1) and the water-soluble homogenates of cave controls contained more PCP than all other groups (Table B.1)

A complex profile of methanol soluble pigments from tissue and skeletal material were separated by C8 reverse phase chromatography. Pigments were identified by comparison to co-eluted standards and/or by to photodiode array spectra. Pigments identified included chl *a, b* and c_2, xanthophylls cycling pigments diadinoxanthin, and diatoxanthin, the dinoflagellate specific pigments peridinin, and the siphonous green algae specific pigment siphonein (Fig. B.3). Chl *a* resulting from both dinoflagellates and endolithic algae was distinguished into three peaks chl *a*

allomer, chl *a* and chl *a* epimer. Each of these three peaks had characteristic chl *a* spectrum and was named according to the elution sequence observed in Zapata et al. (2000). A chl *a* standard treated similarly to the biological samples eluted as a single peak (data not shown).

Chl c_2 and peridinin measured as peak area normalized to surface are at significantly lower concentrations in corals heated to 32°C in comparison to controls maintained at ambient temperature (Fig. B.4B, D). Peridinin concentrations were greatest in the cave-dwelling morph (SNK, $p = 0.003$, Fig. B.4A). In the case of peridinin, there was no interactive effect between temperature and color morph (temperature x morph: $F_{2, 12} = 0.81$, $p = 0.47$). There was however a significant interaction between temperature and morph for chl c_2 (Fig. B.4C). This interaction was due to a greater concentration of chl c_2 in cave-dwelling control corals (SNK, $p = 0.000$, Fig. B.4C) with no difference amongst cave-dwelling heated corals and purple or brown corals from either the controls or heated treatment.

Chl *b* and siphonein are pigments associated with coral endolithic algae, rather than dinoflagellates (Highland 1981). Neither of these pigments when normalized to surface area was affected by temperature (chl *b*, $F_{1,1)} = 1.13$, $p = 0.31$; siphonein, $F_{1,12} = 0.15$, $p = 0.71$), morph (chl *b*, $F_{2,12} = 0.70$, $p = 0.52$; siphonein, $F_{2,12} = 0.72$, $p = 0.51$), or the interactive effect of these factors (chl *b*, $F_{2,12} = 0.25$, $p = 0.78$; siphonein, $F_{2,12} = 0.14$, $p = 0.87$). Based on these results we feel justified in assuming that endolithic algae provide a morph and temperature invariant background contamination to dinoflagellate Chl *a* measurements. Changes in Chl *a* are therefore attributed to dinoflagellates.

Diadinoxanthin (Dd) is de-epoxidated to become diatoxanthin (Dt) in dinoflagellates. Diatoxanthin quenches excitation energy within the antenna of PSII. The dinoflagellates in the cave-dwelling morph had significantly a greater xanthophyll pool (Dd +Dt) than those in the

purple-HL morph (SNK $p = 0.046$, Fig. B.5C). Similarly, dinoflagellates in the purple-HL morph had more Dd + Dt than the brown-HL morph (SNK, $p = 0.041$, Fig. B.5C). Heating corals to 32^{o}C for 6 h resulted in a significant decrease in the concentration of Dd + Dt (Fig. B.5D), the rate of decrease across all morphs was constant as there was no significant interaction between treatment and morph (temperature x morph: $F_{2, 12} = 0.60$, $p = 0.57$). Whilst the pool of xanthophylls decreased with heat, a higher proportion of the remaining pool was in the de-epoxidated form of diatoxanthin (Fig. B.5B). No significant interaction between morph and temperature was observed (temperature x morph: $F_{2, 12} = 0.84$, $p = 0.47$). The brown-HL morph however, had the lowest mean proportion of diatoxanthin in the control and the highest mean proportion of this pigment in the heated treatment (Fig. B.5A).

Total chlorophyll was determined by adding the area under chromatogram peaks for chl *a* allomer, chl *a* and chl *a* epimer. The cave-dwelling morph was found to have more total chl *a* than the purple-HL morph (SNK, $p = 0.021$, Fig. B.5E). The brown-HL morph contained an intermediate, but not significantly distinct concentration of total chl *a* (Fig. B.5E). Again, temperature was found to negatively effect the concentration of total chl *a* (Fig. B.5F), and the lack of interaction between temperature and morph suggested that the rate of decrease was constant across all morphs (temperature x morph: $F_{2,12} = 1.82$, $p = 0.20$). The proportion of chl *a* allomer to total chl *a* was also analyzed. A significantly greater proportion of chl *a* allomer was extracted from corals held at elevated temperature (Fig. B.5H). Again the cave-dwelling morph contained significantly more of this oxidized form of chl *a* than the purple-HL morph (SNK, $p = 0.021$, Fig. B.5G), with the brown-HL morph containing an intermediate, but not significantly different concentration of chl *a* allomer (Fig. B.5G).

The relative size of the xanthophylls pool was less for the heated treatment than for controls (32^{o}C, (Dt + Dd) Chl a^{-1} = 0.21; control, (Dt + Dd) Chl a^{-1} = 0.23). These results show that there was no expected increase in the relative xanthophyll pool in response to a temperature stress. The relative size of the xanthophyll pool was greater for the purple-HL morph (= 0.39) than for the brown-LL morph (= 0.24), the brown-HL morph (= 0.10) had the lowest xanthophyll pool size relative to Chl *a*. Suggesting that Dt +Dd normalized to Chl *a* did not relate to the light habitat from which the corals were obtained. Interactive relative xanthophyll pool means were not compared as there was no interactive effect for either Dt + Dd or Chl *a* concentrations.

Discussion:

Why did Montipora monasteriata look bleached after heating to 32 ^{o}C for 6 h?

Bleaching, more often than not is reported and discussed only in terms of an actual loss of dinoflagellates (eg., Coles and Brown 2003; Tchernov et al. 2004). This has led many studies to assume that visible losses of pigmentation in corals are necessarily caused by a loss of resident dinoflagellates (eg. Baker 2001). The sometimes untested assumption is then used to spear head the argument that bleaching is required to open up residential space within the coral house for occupation by a "new" dinoflagellate population (Baker 2001; Buddermeir and Fautin 1993). In this study, a reduction in chlorophyll concentration associated with heat stress was observed across all morphs under relatively low external light intensities (< 650 μmol quanta m^{-2} s^{-1}, but averaging only 50 μmol quanta m^{-2} s^{-1}) in the absence of significant dinoflagellate loss. Decreases in Chl *a* concentrations without dinoflagellate reductions have previously been reported by a number of studies investigated the effects of increased photon flux (eg., Falkowski and Dubinski 1981; Hoegh-Guldberg and Smith 1989). Experimental studies on thermal

bleaching have tended to investigate the effect of heat on dinoflagellate populations, Chl fluorescence parameters and only occasionally Chl concentrations, mostly Chl *a* as opposed to Chl c_2 and never carotenoids. The results of these studies are varied even so for so called "bleaching sensitive corals" with some reporting 50 % or greater loses of dinoflagellates for visibly bleached corals (eg. Hoegh-Guldberg and Smith 1989; after 4 d of heating to 32^{o}C) and others reporting losses of Chl *a* as opposed to dinoflagellates (eg. Takahashi et al. 2004; after 12 h of heating to 32 ^{o}C). These experiments, for the most part, are performed with different coral species, differently pigmented morph and under different light conditions. Takahashi et al. (2004) results were obtained under a light regime that was unnatural, at a constant 1000 μmol quanta m^{-2} s^{-1} PAR for 12 h, even for shallow water corals (1 – 3 m). The data presented in this study however, show that loss of Chl *a* without loss of symbionts also occurs when corals are subjected to elevated temperature in conjunction with PAR regimes equivalent to or less than the photon flux density (PFD) experienced in their natural habitat.

In higher plants, the major light harvesting Chl proteins of PSII (LHCP II) and early light inducible proteins (ELIPs), a subgroup of LHCPs that bind xanthophylls-cycle carotenoids in addition to Chl, have a reciprocal response to changes in the excitation pressure on PSII (Krol et al. 1995). ELIP mRNAs are expressed within 2 h under increased PFD (Krol et al. 1995), with increases in ELIP protein noted within a single day (Montané et al. 1998). The rapid response is partially owed to ELIPs scavenging components from degrading LHCP II. In the present study, Chl loss (Chl *a* and Chl c_2) does not appear to be a rapid acclimation to an increase in PSII excitation pressure because the abundance of xanthophyll-cyclic carotenoids were simultaneously reduced by the application of heat. Dinoflagellates are photosynthetically unique in so far as their light harvesting antennae is formed from water soluble peridinin-chl a-proteins

(PCPs) and chl a-chl c_2-peridinin-protein complexes (acpPCs; aka, *i*PCPs). The peripheral antennae (PCP) are unrelated to the family of known LHCP. The membrane bound acpPCs, on the other hand, are related to the LHCP family of proteins (Green and Durnford 1996). Iglesias-Prieto and Trench (1997) demonstrated that some, but not all, cultured dinoflagellates belonging to the genus *Symbiodinium* are able to respond to increases in PFD by increasing their xanthophylls pool. This ability is thought to be associated to a differential ability to express appropriate acpPC genes (Iglesias-Prieto and Trench 1997).

M. monasteriata looked bleached after heating to 32 ^{o}C for 6 h because they have lost 50 % or more of their total chlorophyll pool, peridinin and xanthophyll pool. The reduction of the xanthophyll pool is of particular interest as it does not conform to typical photoacclimatory behaviour amongst photosynthezing organisms, inclusive of some *Symbiodinium sp*. Is the lack of a relative increase in xanthophylls pool due to: temperature stress providing an inappropriate signal for photo-acclimation; the presence of inappropriate dinoflagellates; and/or after 6 h of heat, are the photosynthetic units of these dinoflagellates undergoing rampant photo-oxidative damage?

Does a temperature- initiated-excitation-pressure on PSII provide an inappropriate signal for xanthophylls accumulation?

Excitation pressure on PSII due to elevated thermal stress can, as discussed in the introduction, eventuate for a number of different reasons other than increasing PDF. Excitation pressure on PSII will increase as a result of interference in electron transport and due to limited access to final electron transport acceptors. In higher plants, it appears that increased photon flux is required for a photo-acclimation response at the level of proteins and pigments (Montané

et al. 1998), whereas in unicellular algae a non-PFD driven increase in excitation pressure is capable of stimulating structural charges to the antennae (Maxwell et al. 1995). The fact that higher plants seem to respond distinctly to different environmental cues, whereas unicellular algae appear to have a single response to a variety of environmental cues channeled through the redox state of the photosynthetic apparatus has been attributed to the complexity of higher plant physiology (Walters 2004). Studies have shown that for cultured unicellular *Symbiodinium sp*., tolerance to high PFD does not automatically confer tolerance to high temperature (eg. *Symbiodinium microadriaticum* from the scyphozoan, *Cassiopeia sp.*; Iglesias-Prieto et al. 1992; Iglesias-Prieto and Trench 1997). Results such as these would seem to suggest that free living *Symbiodinium* respond distinctly to different environmental cues, emulating in this respect higher plants rather than unicellular colleagues such as green algae from the genus *Chlorella*. Perhaps the distinction is attributable to the dominant life style of each organism. In contrast to *Chlorella*, *Symbiodinium sp.* predominantly occurs in symbiosis with a wide variety of phyla (Taylor 1973; Trench 1979). The physiology of the holosymbiont is anything but simple. This is amply demonstrated by the present study were no single uniform host response to temperature was observed for either of the two parameters measured. As a result of heating to 32^{o}C, host pigmentation in purple-HL morphs was reduced by 75 %, whereas the brown-HL morph, that lacked host pigmentation, experienced a 50 % reduction in host protein concentration, and the brown-LL morph that also lacked host pigmentation, experienced no significant reduction in host protein. Significantly, dinoflagellates residing within the least host affected brown-LL morph were less photoinhibited, according to dark-adapted F_v/F_m measurements, than more host affected purple-HL and brown-HL morphs. This is a counterintuitive results given the indication based on elevated control Chl c_2 and PCP concentrations that this cave dwelling morph was

initially more shade-acclimated, than either the purple-HL or the brown-LL morphs. Typically, shade-acclimated corals have lower saturation irradiances I_k and photosynthetic maxima, P_{max} (Falkowski and Dubinski 1981, Anthony and Hoegh-Guldberg 2003), with the consequence that brown-LL morphs should be nearer to their excitation pressure threshold than either purple-HL and brown-HL morphs. A potential solution to the paradox driven by differential changes in tissue thickness (*aka* protein content), pocilloporin and dinoflagellate pigmentation is that the difference in light-dose per unit Chl *a* between control and heated cave-dwelling morphs, may be less than that experienced by open-dwelling morphs (Enríquez et a. 2005). Alternatively, it may be due to the fact that the C21 *Symbiodinium sp.* found to be residing in the brown-LL morph are more heat tolerant than the C17 *Symbiodinium sp.* resident in open-dwelling morphs.

How flexible are these different morphs at handing either high PDF or elevated temperature?

In contrast to the results of this study, Anthony and Hoegh-Guldberg (2003) found no difference in the resident symbiont type (all were C17 for *M. monasteriata* collected from open or cave habitats. In Anthony and Hoegh-Guldberg (2003), C17 dinoflagellates showed a high capacity to acclimate (in 3 weeks) to downshifts or upshifts in irradiance. This flexibility to accommodate variable PFDs has been found in cultured *Symbiodinium sp* to correlate with the ability to express both monomeric and dimeric forms of PCP, enrichment in the monomeric form occurs under high PFD and coincides with an increase in the xanthophyll pool (Iglesias-Prieto and Trench 1997). In this study, PCP from both C17 and C21 eluted with red absorption maxima at 670.6 nm and 671.7 nm respectively that are indicative of a mixing of monomeric and dimeric forms, and suggest that both C17 and C21 dinoflagellates are able to flexibly acclimate to different light regimes (Iglesias-Prieto and Trench 1997). Based on this circumstantial

evidence it does not seem as though either of these dinoflagellates was incapable of responding to elevated PFD by increasing their xanthophylls pool.

Recently Tchernov et al (2004) have supported the view that *Symbiodinium sp.* thermostability is dictated by the polyunsaturated fatty acid composition of the thylakoid membrane (Iglesias-Prieto et al. 1992, Tchernov 2004). In this context, sensitivity to thermal bleaching was based singly on the predisposition of corals to shed dinoflagellate cells. Corals containing sensitive dinoflagellates shed their symbionts after only 3 d at 32^{o}C, whilst bleaching insensitive species retain symbionts for greater than 2 months at this temperature. Based on a comparison of cultured thermally tolerant an intolerant *Symbiodinium sp*., bleaching was argued not to eventuate due to excess excitation pressure on PSII, but rather due to a thermal meltdown in the integrity of the thylakoid membrane that is then unable to form the necessary proton gradient to drive both assimilatory photochemical quenching and non-photochemical quenching, thereby forcing excitation energy through the water-water cycle and risking the generation of super oxide anions (O_2^-) and hydroxyl radicals (OH,). According to the model presented, it is the leaking of these reactive oxygen species (ROS) to the host that leads to their expulsion (Tchernov et al. 2004). This non-photoinhibition model of bleaching is very elegant and attractive in so far as it efficient captures the notion that dinoflagellates can handle high PFD, but fail to handle elevated temperature. Its consistency however with other studies conducted on thermally sensitive *Symbiodinium* cultures at 32^{o}C under similar PDF is questionable: For example, Iglesias et al. (1992) noted a subtle decrease in the rate of electron transfer between the Q_A and Q_B and hence increase in excitation pressure at PSII after just 45 min at 32^{o}C for *S. microadriaticum*. Warner et al. (1999) noted increasing non-photochemical quenching (qN) for a cultured thermally sensitive dinoflagellate 7 d into heating to 32^{o}C. This latter study is

particularly significant because the Tchernov et al. (2004) Chl fluorescence data, upon which the conclusion was based, was also collected 7 d into heating.

The significant visual paling observed to occur 6 h into heating to 32^{o}C in this study falls outside the definition for bleaching assumed by Tchernov et al (2004). In the present study, the visual paling observed mirrors the rapid photo-bleaching observed to occur in algae and plants that are that are exposed to increased PDF but deficient in carotenoids (Nigoyi 1999). The increase in Chl *a* allomerisation witnessed for thermally stressed corals is consistent with a photoinhibition model of thermal bleaching where antennal Chl is unable to pass on excitation energy to similarly energised neighbours. Allomerisation of chl *a* occurs when the enolate anion of chl *a* is oxidized by free radicals (Hynninen 1991). In vitro, it is inhibited in the dark by the presence of β-carotene (Hynninen 1991) which is believed to primarily scavenge $^{1}O_2$ (Bumann and Oesterhelt 1995) and hence although alternative models for allomerisation have been presented it is thought to be principally driven by $^{1}O_2$ as opposed to other reactive oxygen species. Also consistent with a photoinhibition model of thermal bleaching is the relative increase in non-photochemical quenching observed as an increase in Dt/(Dd+Dt) for the heated treatment. Increased ΔpH across the thylakoid membrane is required for the stimulation of Dd de-epoxidase activity, and a down-regulation of Dt epoxidase activity. Increases in Dt/(Dd + Dt) cannot occur over a leaky thylakoid membrane (Mewes and Richter 2002). In higher plants, it has been demonstrated that zeaxanthin, the analogue of dinoflagellate Dt, incorporates into the lipid bilayer stabilizing increased thylakoid membrane fluidity induce by thermal stress (Harvaux and Tardy 1996). An active xanthophyll cycle therefore performs at least two essential functions under heat stress: a non-photochemical quenching and a membrane stabilization role. Singlet oxygen is not only capable of photo-bleaching antennae Chl (Thompson and Brudvig 1988), but

also P680 in the reaction centre of PSII (Telfer et al. 1994). Liu et al. (2004) have recently argued that under high PFD O_2^- is generated defensively in preference to the more harmful $^1O_2$. This is a high-risk defense mechanisms that in the presence of an inadequate antioxidant system can lead to the formation of hydrogen peroxide and OH radicals and a subsequent peroxidation of membrane lipids.

Bleaching (loss of pigmentation) in the case of *M. monasteriata* clearly preceded total loss of thylakoid membrane integrity and clearly occurred due an increase in the excitation pressure on PSII. A failure to increase the relative size of the xanthophylls pool may have played a significant additional role in the observed photo-oxidation of the light harvesting antennae and also via a loss of their stabilizing role may eventually lead to thylakoid membrane meltdown. The heating experiment was terminated at 6 h and hence we have no idea as to whether bleaching (loss of dinoflagellates) would eventuate for any of the experimental morphs under investigation. Enriquez et al. (2005) have recently demonstrated that significant loss of Chl can led to an exponential increase in the path length of light within coral tissue. Under such a scenario, high PFD, as well as high thermal tolerance may be required of resident dinoflagellates; the host's ability to withstand UV damage and mop up reactive oxygen species would also take on a new significance.

Table B.1 Multivariate Wilk's lambda 2-factorial analysis of variance testing the effect of elevated temperature (temp) and morph on PCP and pocilloporin pigmentation determined by area under 580 nm and 420 nm gel filtration chromatograms.

Factor	Value	*F*	Effect (df)	Error (df)	*p*	post-hoc (SNK)
temp	0.297	13.033	2	11	0.00125	PCP: control > $_{0.007}$ heated; Pocilloporin: control >$_{0.0006}$ heated
morph	0.044	20.666	4	22	0.00000	PCP: cave >$_{0.03}$ others; Pocilloporin: purple >$_{0.0002}$ others
Temp x morph	0.185	7.277	4	22	0.00068	PCP: control cave >$_{0.015}$ others; Pocilloporin: control purple>$_{0.0002}$ others

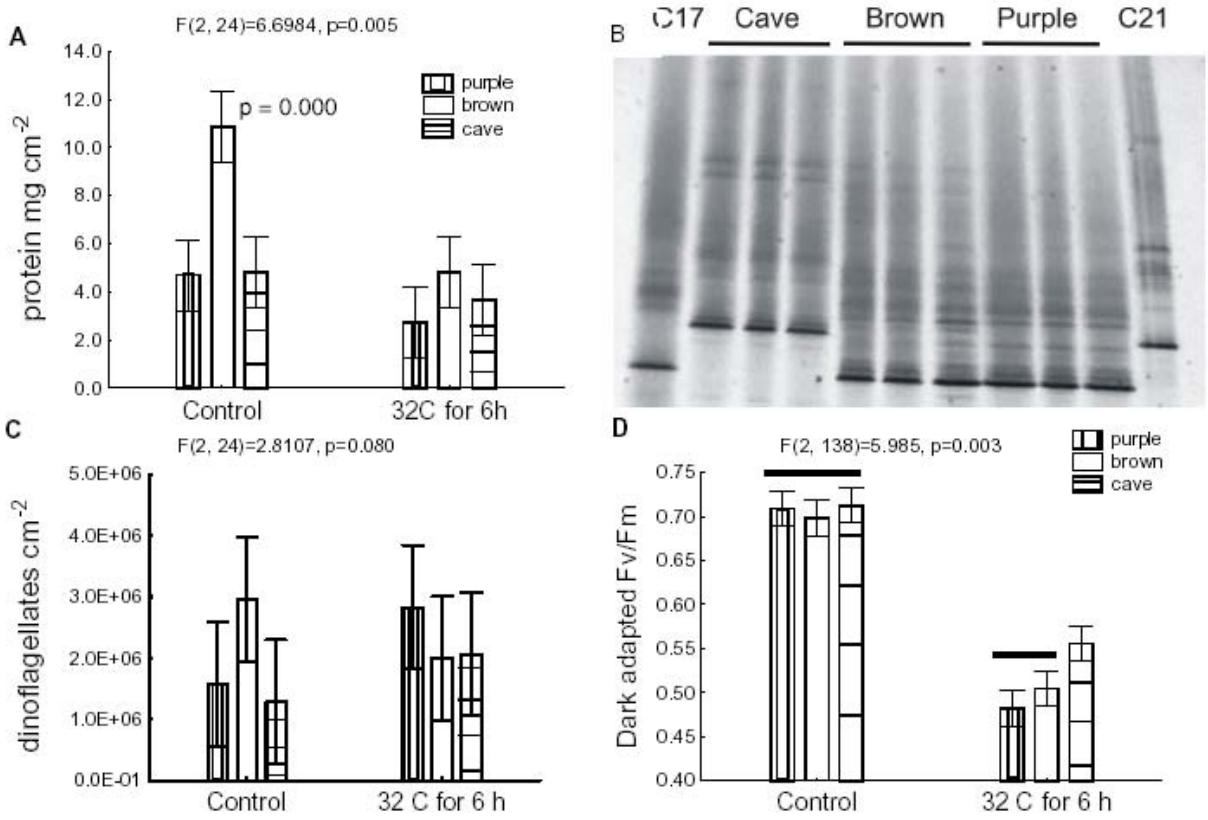


Figure B.1 (A) Summary of results obtained for 2-Factorial ANOVA the interactive effect of morph and temperature on the water-soluble protein concentration of host homogenates normalized to coral surface area. *p*-values for SNK post-hoc analyses distinguishing brown-HL control from other groups is shown. (B). DGGE Profile comparison of dinoflagellates residing in distinct morphs to standard C17 and C21 dinoflagellate subclades. (C). Interactive effect of morph and temperature on symbiotic dinoflagellate density normalized to surface area. (D). Interactive effect of morph and temperature on dark-adapted Fv/Fm. *Lines* link groups that do not significantly differ from each other in an SNK analysis. Error bars represent 95% confidence intervals. *Cave*, Brown-LL; *brown*, brown-HL; *purple*, purple-HL.

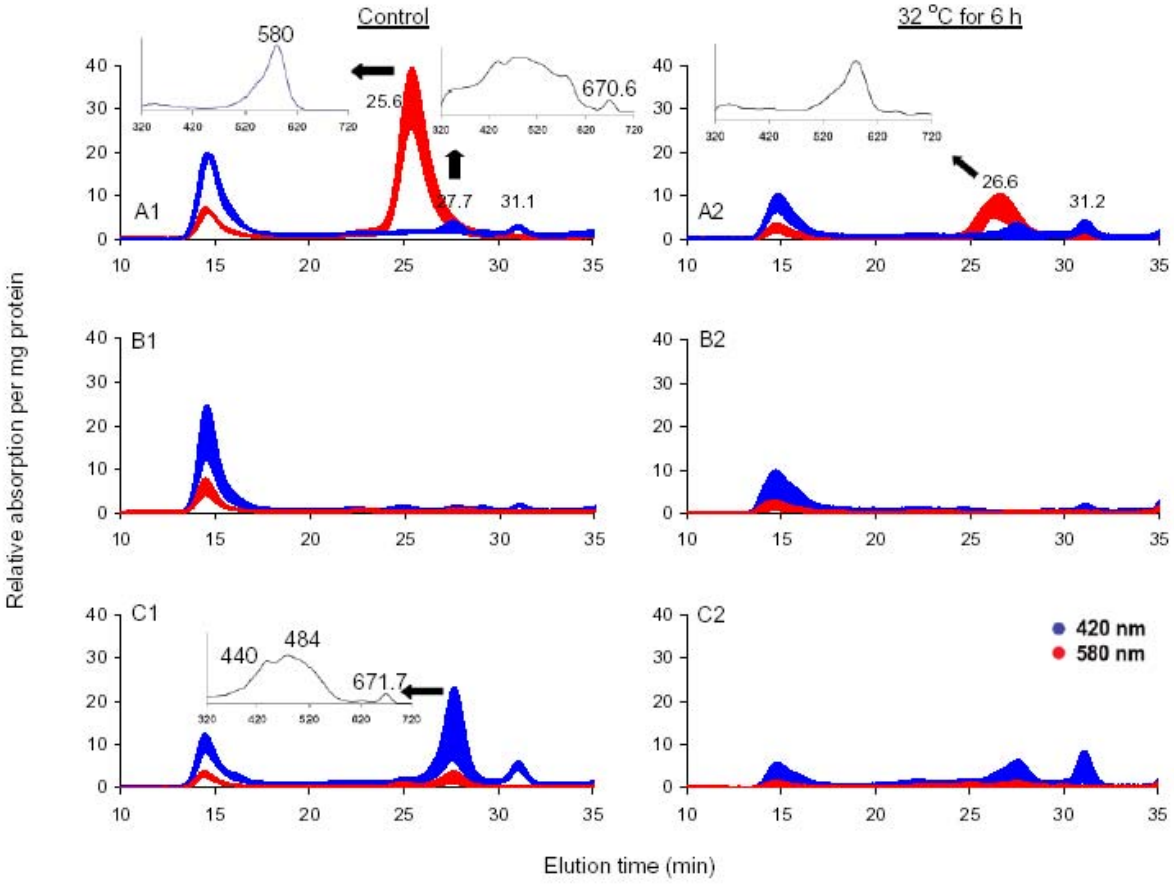


Figure B.2 Gel filtration chromatograms of water soluble homogenate monitored at 420 nm and 580 nm. Line width corresponds to mean (± 95% confidence interval) absorption normalized to total soluble protein concentration. Arrows point to inset absorption spectra determined by photodiode array at elution times identified for chromatogram peaks. (A, B) control and heated purple-HL open dwelling morph respectively. (C, D) control and heated brown-HL open dwelling morph respectively. (E, F) control and heated cave-dwelling (brown-LL) morph respectively.

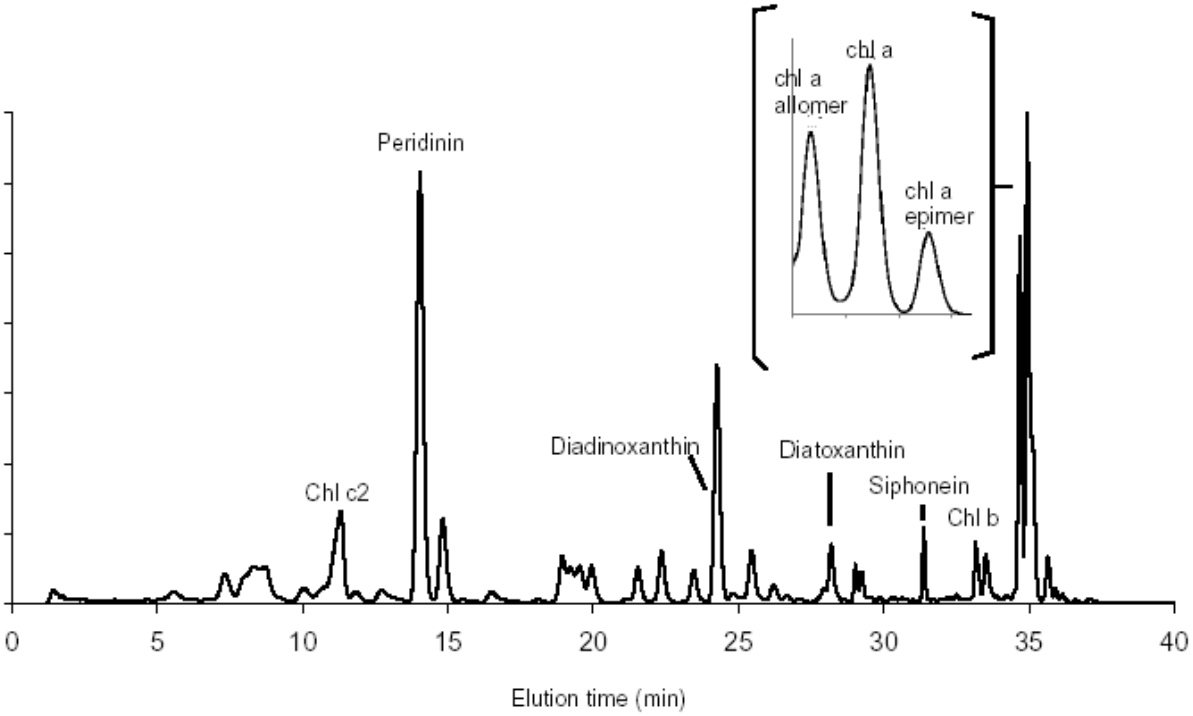


Figure B.3 Typical C-8 elution profile monitored at 450 nm for methanol-soluble pigments extracted from whole coral fragments using the methodology of Zapata et al. (2000).

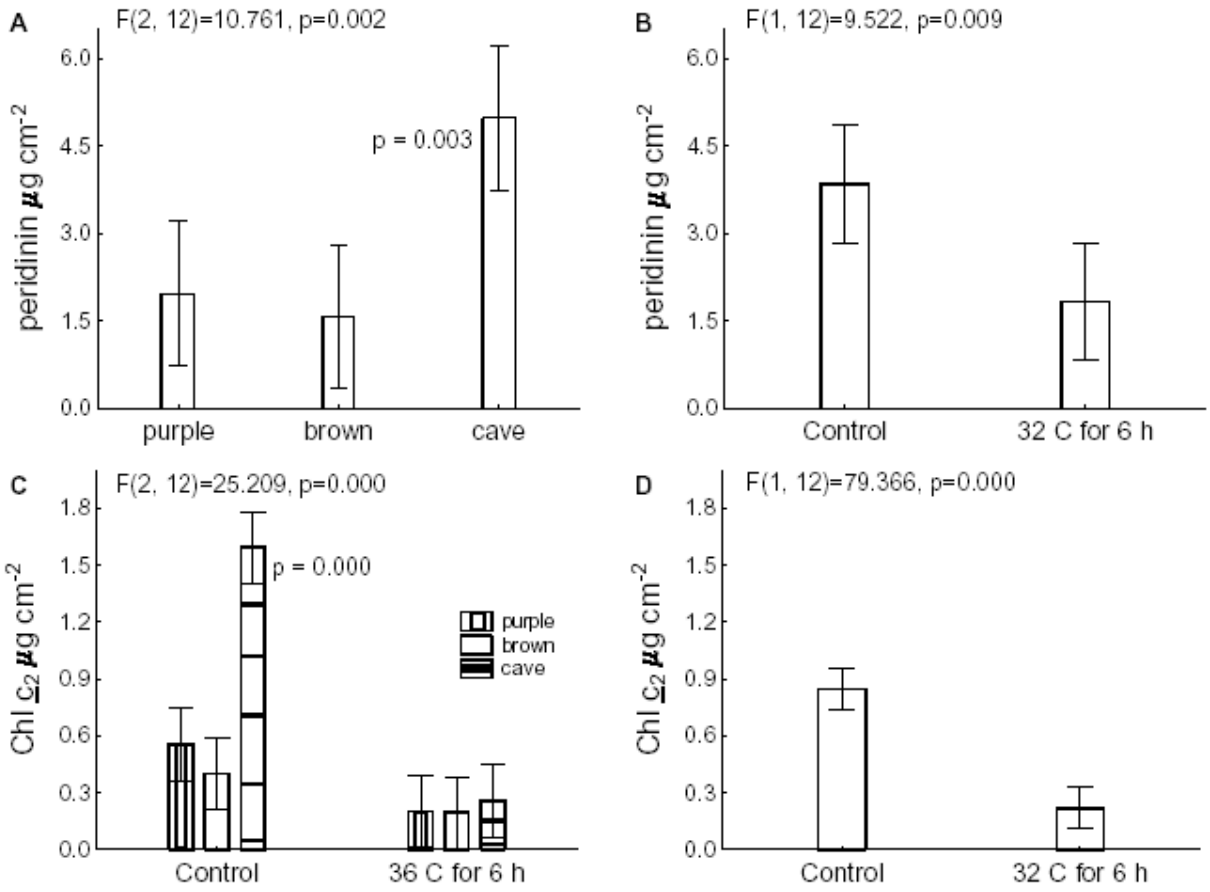
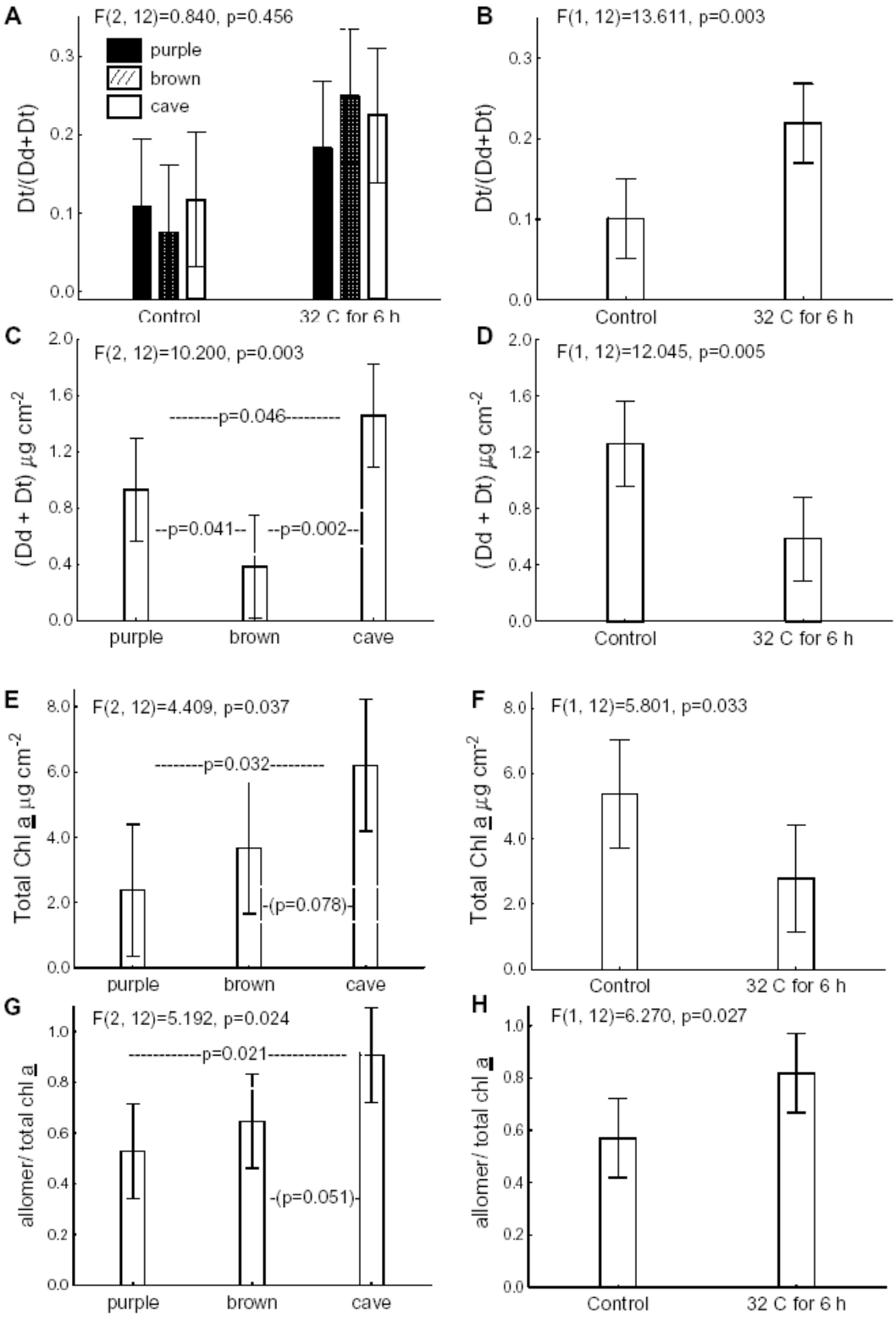


Figure B.4 Summary of significant results obtained for a 2-Factorial ANOVA determining the effects of morph and temperature treatment on dinoflagellates peridinin and Chl $\underline{c_2}$ concentrations normalized to surface area: (A) Effect of morph on peridinin concentration. *p*-values for SNK post-hoc analyses distinguishing cave from other morphs is shown. (B) Effect of temperature on peridinin concentration. (C) Effect of the interaction between morph and temperature on Chl $\underline{c_2}$ content. *p*-values for SNK *post-hoc* analyses distinguishing cave control from all other groups morphs is shown. (D) Effect of temperature on Chl $\underline{c_2}$ content. Error bars represent 95% confidence intervals. *Cave*, Brown-LL; *brown*, brown-HL; *purple*, purple-HL.

Figure B.5 (A-D) Summary results obtained for a 2-Factorial ANOVA determining the effects of morph and temperature treatment on dinoflagellates xanthophyll content and (E-H) on algal Chl *a* content. Xanthophyll cycling Dt(Dd+Dt) was not significantly affected by morph or by the interaction of (A) morph and temperature, but was significantly defected by (B) temperature. Dd+Dt normalized to surface area gave no interactive effect, but was significantly affected (C) by morph and (D) by temperature. Total Chl *a* content normalized to surface area gave no interactive effect, but was significantly effected by (E) morph and (F) temperature. The ratio of allomerised Chl *a* to total Chl *a* gave no interactive effect, but was significantly effected by (G) morph and (H) temperature. SNK *p*-values distinguishing morphs are shown, bracketed values are not significant. Error bars represent 95% confidence intervals. *Cave*, Brown-LL; *brown*, brown-HL; *purple*, purple-HL.


A
F(2, 12)=0.840, p=0.456
purple
brown
cave
Dt/(Dd+Dt)
0.0
0.1
0.2
0.3
Control
32 C for 6 h
B
F(1, 12)=13.611, p=0.003
Dt/(Dd+Dt)
0.0
0.1
0.2
0.3
Control
32 C for 6 h
C
F(2, 12)=10.200, p=0.003
--------p=0.046---------
--p=0.041--
--p=0.002--
(Dd + Dt) µg cm-2
0.0
0.4
0.8
1.2
1.6
2.0
purple
brown
cave
D
F(1, 12)=12.045, p=0.005
(Dd + Dt) µg cm-2
0.0
0.4
0.8
1.2
1.6
2.0
Control
32 C for 6 h
E
F(2, 12)=4.409, p=0.037
--------p=0.032---------
-(p=0.078)-
Total Chl a µg cm-2
0.0
2.0
4.0
6.0
8.0
purple
brown
cave
F
F(1, 12)=5.801, p=0.033
Total Chl a µg cm-2
0.0
2.0
4.0
6.0
8.0
Control
32 C for 6 h
G
F(2, 12)=5.192, p=0.024
----------p=0.021----------
-(p=0.051)-
allomer/ total chl a
0.0
0.2
0.4
0.6
0.8
1.0
purple
brown
cave
H
F(1, 12)=6.270, p=0.027
allomer/ total chl a
0.0
0.2
0.4
0.6
0.8
1.0
Control
32 C for 6 h

APPENDIX

C- PATTERNS OF INORGANIC PHOSPHATE UPTAKE IN *CASSIOPEA XAMACHANA*: A BIOINDICATOR SPECIES[8]

[8] Todd BD, Thornhill DJ, and WK Fitt. In submission to *Bulletin of Marine Science*.

Abstract

Nutrient levels in the nearshore waters of the Florida Keys have increased over the past few decades concomitant with a decline in the health of Florida's reef system. Phosphorus is a particular concern in the Florida Keys as it may be the limiting nutrient in nearshore waters. We demonstrate that the upside-down jellyfish, *Cassiopea xamachana*, decreases its rate of phosphate uptake following exposure to elevated levels of dissolved inorganic phosphate. We also show that this subsequent suppression of uptake rates persists for some time following exposure to elevated phosphates. Using these attributes, we experimentally investigated the use of *C. xamachana* as a bioindicator for dissolved inorganic phosphates in seawater. Our results show that these animals reveal comparative differences in environmental phosphates despite traditional testing methods yielding no detectable phosphates. We propose that *C. xamachana* is a bioindicator useful for integrating relevant information about phosphate availability in low nutrient environments.

Introduction:

Florida's coral reef ecosystem is declining in coral cover and is at risk for further degradation (Porter and Meier, 1992; Porter et al., 2002). One cause for the decline is increased turbidity and nutrient loading resulting from interconnectedness between the offshore reefs and nearshore waters, including Florida Bay (Porter et al., 1999; Cook et al., 2002). Elevated nutrients are implicated in the localized deterioration of coral reefs and they act together with other stressors to contribute to the decline of coral reefs globally (Szmant, 2002). Increased nutrient inputs reduce coral growth and reproduction and contribute to macroalgae proliferation

and overgrowth, leading to changes in reef community structure (Simkiss, 1964; Hughes, 1994; Koop et al., 2001; Loya et al., 2004).

The source of rising nutrients in the Florida Keys remains unresolved (Boyer and Jones, 2002; Lapointe et al., 2002). Nutrients are intimately associated with sewage and the dissolved organic forms found in sewage are easily remineralized to more readily available inorganic forms favored by plants and algae (Szmant, 2002). Shinn et al. (1994) showed that injected wellwater rapidly migrates both vertically and horizontally in porous Florida limestone; nutrient-rich contaminated groundwater seeps into coastal and ocean waters. Viral tracer studies corroborate this rapid movement of wastewater from on-site sewage disposal systems toward the coral reef systems in the lower Florida Keys (Paul et al., 2000). Additionally, Patterson et al. (2002) reported that the white pox coral disease afflicting the Elkhorn Coral, *Acropora palmata*, is caused by *Serratia marcescens*, a bacterium commonly found in human intestines. However, other authors offer the contrasting evidence that elevated nutrient levels found on reefs are likely not a result of shore-based or Florida Bay-influenced sources, but instead derive from resuspension of nutrient-rich sediments by offshore upwelling (Szmant and Forrester, 1996; Leichter, 2003). Regardless of the source, it is important to understand the distribution and impact these nutrients have on reefs in the Florida Keys.

Phosphorus is a particular concern in the Florida Keys as it may be the limiting nutrient in nearshore waters (Lapointe, 1989; Cook et al., 2002). However, if uptake of phosphates by the surrounding biota is rapid, then measurement of nutrient concentrations alone may not adequately assess nutrient availability in the habitat (Koop et al., 2001). Thus, a potentially more informative way of monitoring for increased environmental nutrients is through the use of bioindicators. Bioindicators are commonly used for detecting *in situ* pollution or the extent of

anthropogenic influence on an environment. Examination of stable isotope ratios of $\delta^{15}N/\ \delta^{14}N$ in reef organism tissues is one method of determining the level of human sewage inputs to reef systems (Yamamuro et al., 2003). Other analyses that rely on bioindicators to determine relative nutrient enrichment include examining chlorophyll *a* activity in coastal waters (Harding and Perry, 1997), detecting alkaline phosphatase in phytoplankton (Lapointe, 1989), and assaying seaweeds and seagrasses to determine nutrient enrichment and limitation in surrounding seawater (Lapointe et al., 1994).

Cassiopea xamachana is a jellyfish symbiotic with zooxanthellae, unicellular dinoflagellates of the genus *Symbiodinium* (Freudenthal, 1962). Like other symbiotic cnidarians, these jellyfish acquire dissolved nutrients from the surrounding seawater to meet the needs of their photosynthetic partners (Pomeroy and Kuenzler, 1969). Additionally, high levels of available nutrients have been shown to negatively affect the subsequent nutrient uptake rates by both host animals and the symbionts (Jackson and Yellowlees, 1990; Belda and Yellowlees, 1995; Kelty and Lipschultz, 2002). We tested the hypothesis that exposure to elevated dissolved inorganic phosphate (DIP) causes a decrease in the subsequent uptake rate of *C. xamachana.* In a second experiment, we used lower concentrations of elevated DIP in the experimental seawater, but increased the length of time that *C. xamachana* were exposed to the solutions in order to test the hypotheses that uptake rates would decrease with increasing phosphate concentrations and increasing lengths of exposure to those concentrations. We performed a third experiment to determine the length of time that reduced uptake rates would persist following exposure to elevated DIP. We performed our fourth experiment to demonstrate the application of *C. xamachana* as a bioindicator of DIP in seawater by transplanting the animals to local reef environments and comparing subsequent uptake rates following four days of exposure.

Methods:

Animal collection

Cassiopea xamachana were collected from a nearshore mangrove lagoon on the Atlantic coast of Key Largo, FL (24˚59'N, 80˚22'W) near highway US 1 mile marker 99.5. This mangrove lagoon is ocean fed from both ends, allowing tidal currents to enter and exit. Ambient levels of DIP were typically less than 0.1 μM as measured using the methods described below. All animals were collected within a few meters from each other with the assumption that they had a shared nutrient history. Animals were transported to the lab in water collected at the site and used in experiments within the hour unless otherwise indicated. Medusae were selected within a narrow size range (4.5 – 6.0 cm bell diameter) in order to reduce size-related variability in uptake rates. Bell diameters were recorded to the nearest tenth of a centimeter while extended. Animals were blotted dry once and wet weights were obtained by weighing on a Metler scale to the nearest hundredth of a gram.

Quantifying phosphate uptake rates

Phosphate uptake rates for cnidarians are typically calculated as the rate of phosphate depletion from seawater (Pomeroy and Kuenzles, 1969; D'Elia, 1977; Belda and Yellowlees, 1995). These methods were followed with minor modifications as described below. *Cassiopea xamachana* jellyfish were placed into acid-washed glass Petri dishes of adequate size so that the bell of the animal could completely extend. Each Petri dish contained 200 ml of Instant Ocean seawater (35 ppt). The seawater was mixed to contain approximately 2.0 μM phosphate in the form of dissolved KH_2PO_4. This concentration was chosen to minimize the likelihood that an animal would completely deplete all DIP from the seawater before the end of the ten-minute

assay. Three 50 ml samples of the starting solution were collected to determine the precise initial concentration for the mixture. Animals were allowed to deplete the dissolved KH_2PO_4 from the medium for only ten minutes to minimize the likelihood that exposure to the 2.0 μM DIP assay solution would affect the final calculated uptake rates. Duplicate 25 ml water samples were collected at the conclusion of the assay and all samples were immediately placed in a -20°C freezer. Less than 24 hours later, samples were thawed and prepared for DIP determination using the addition of a molybdate compound and following the heteropoly blue formation method (Strickland and Parsons, 1972). The change in the concentration of DIP over ten minutes was used to determine the uptake rates of the animals.

Experiment 1: Exposure to elevated DIP for one hour

This experiment was conducted to test the hypothesis that exposure to elevated concentrations of DIP in seawater causes a reduction in the phosphate uptake rates of *Cassiopea xamachana*. Eighteen *C. xamachana* were collected and randomly divided into six equal groups of three animals. Each group was then exposed to a different concentration of dissolved KH_2PO_4 in Instant Ocean seawater (35 ppt) for one hour. The six predetermined concentrations were 0, 0.2, 0.5, 1.0, 2.0, and 20 μM DIP. These values encompass known DIP concentrations on eutrophic reefs as well as concentrations that have been shown to reduce phosphate uptake in a clam-zooxanthellae interaction (Belda and Yellowlees, 1995; Szmant and Forrester, 1996; Szmant, 2002). Phosphate concentrations in the salt mix are known to be negligible and were undetectable by our methods (<0.03 μM). Specimens were placed in large glassware and kept outdoors in a naturally lit area at 25 ± 1 °C. Animals were incubated in their respective solutions for one hour and then removed. All specimens were rinsed gently in filtered seawater to prevent phosphate carry-over from incubation solutions. Assays were then performed to quantify the

uptake rates as described above. To test the hypothesis that incubation at elevated phosphate levels for one hour would have an effect on phosphate uptake parameters, data were analyzed using one-way ANOVA (α=0.05) with the analytical software Statistica (Statsoft, 1999). Where a significant effect was found, Fisher's protected least significant difference test was used to identify differences between groups. Tests of the assumptions underlying the analyses were made prior to performing each analysis (Zar, 1998). No serious violations of the assumptions were evident at the level α=0.05.

Experiment 2: Increased length of exposure to moderate levels of DIP

This experiment was conducted to determine if lengthier exposures to moderately elevated DIP levels would negatively affect subsequent uptake rates. Forty-five medusae were collected for the experiment and three separate environmental tanks were established with 0.1, 0.2, and 0.5 μM dissolved KH_2PO_4. These levels are lower than those used in the first experiment and they were chosen because they are more representative of actual phosphate levels recorded in reef environments (e.g., Szmant and Forrester, 1996; Szmant, 2002). The environmental tanks were placed outdoors in a naturally lit area and kept at 25 ± 1 ^{o}C. Repeated addition of KH_2PO_4 to the three tanks throughout the experiment kept phosphate concentrations relatively constant as verified through periodic water testing. The 45 individuals were divided into three equal groups of 15 and randomly assigned one of these tanks. Exposure was initiated at 10:00 AM and three individuals from each tank were removed and assayed to determine uptake rates 1, 3, 6, 12, and 24 hours after placement in the tank. Data were analyzed using two-way ANOVA (α=0.05) with the analytical software Statistica (Statsoft, 1999) to test the hypotheses that DIP uptake rates would decrease with increasing phosphate concentrations and increasing lengths of exposure to elevated phosphates. Where a significant effect was found,

Fisher's protected least significant difference test was used to identify differences between groups. Again, no serious violations of the assumptions were evident at the level $\alpha=0.05$.

Experiment 3: Duration of nutrient uptake signature

Fifteen *Cassiopea xamachana* were collected in the mangrove lagoon 10 m from shore and transported in a cooler filled with water from the collection site to Athens, GA, USA where this experiment was conducted on the following day. *C. xamachana* were kept at 25 ± 1 ^{o}C under constant artificial light for the duration of this experiment. *C. xamachana* were exposed to 2.0 μM dissolved KH_2PO_4 for one hour. Following one hour of exposure, the animals were removed and rinsed gently with artificial seawater to prevent phosphate carryover before being placed into a holding tank of Instant Ocean artificial seawater with no dissolved phosphates. Three animals were immediately used to determine phosphate uptake rates following elevated DIP exposure, and three additional animals were randomly removed 1, 5, 24, and 48 hours later to determine their phosphate uptake rates. Data were analyzed using one-way ANOVA ($\alpha=0.05$) to test the hypothesis that removal from exposure to elevated DIP would result in a return to normalcy of phosphate uptake rates. Where a significant effect was found, Fisher's protected least significant difference test was used to identify differences between groups. No serious violations of the assumptions were evident at the $\alpha=0.05$ level.

Experiment 4: Using Cassiopea xamachana as a bioindicator

Twenty-seven *Cassiopea xamachana* were collected in the mangrove lagoon 10 m from shore and separated into three random groups of equal sample size for transplantation to one of three different environments: 1) Florida Bay 10 m from shore, 2) Admiral Reef, a patch reef approximately 4.8 km offshore, and 3) Little Grecian Reef, a fore reef 8 km offshore. The medusae were contained in clear plastic chambers 10 cm x 10 cm x 15 cm with numerous 1 cm

diameter holes drilled in the sides and screened ends to readily allow exchange of water and exposure to the surrounding environment. Animals were collected after four days and assayed immediately upon collection aboard the boat to determine uptake rates. Forty animals were simultaneously collected from the mangrove lagoon and immediately assayed (< 1 h) to determine reference phosphate uptake rates. Three water samples from each of the four environments were also collected. Phosphate uptake rates were analyzed with one-way ANOVA (α=0.05) to test the hypothesis that uptake rates of animals close to shore would be lower than those of animals farther from shore. Where a significant effect was found, Fisher's protected least significant difference test was used to identify differences between groups. Tests of the underlying assumptions for all analytical procedures were performed prior to analysis and no significant violations were found at the level α=0.05.

Results:

Experiment 1: Exposure to elevated DIP for one hour

Phosphate uptake rates of *Cassiopea xamachana* decreased following exposure to increasing concentrations of DIP (Fig. C.1). Specimens incubated at 20 µM excreted phosphate during the course of the assay while those incubated at 2.0 µM showed no significant active uptake or excretion. Animals incubated at phosphate concentrations less than 2.0 µM took up phosphate during the course of the assay. The uptake rates for the 20 µM, 2.0 µM , and 0.5 µM groups were significantly lower than those from the unexposed control group (*p*-values <0.05). Additionally, uptake rates for animals incubated in 20 µM and 2.0 µM groups were significantly lower than those from all other groups (*p*-values <0.05). The 1.0 µM exposure group did not follow the general trend, apparently due to an anomalous animal or problems with an erroneous measure of one animal.

Experiment 2: Increased length of exposure to moderate levels of DIP

Exposure to elevated phosphates significantly affected the uptake rates of *Cassiopea xamachana* (p=0.042; Fig. C.2). At each interval, jellyfish maintained in 0.5 µM phosphate exhibited uptake rates significantly lower than those of animals incubated at 0.1 µM phosphate (p=0.013) and animals exposed to 0.2 µM had intermediate uptake rates. Uptake rates also varied significantly with length of exposure (p=0.007; Fig. C.2). However, uptake rates did not decrease progressively with corresponding increases in length of exposure as predicted.

Experiment 3: Duration of nutrient uptake signature

Cassiopea xamachana exhibited diminished phosphate uptake rates following one hour of exposure to 2.0 µM dissolved KH_2PO_4, but returned to active uptake within 24 hours (Fig. C.3). The three jellyfish assayed immediately upon removal from the DIP solution had significantly reduced uptake rates compared to jellyfish allowed to recover for 5 or more hours (p-values <0.01). This reduction in uptake rates was still apparent after one hour of recovery when compared to the uptake rates of animals allowed to recover for 24 and 48 hours (p-values <0.05). Major adjustments in the uptake rate occurred in as little as 5 hours after exposure to elevated phosphate.

Experiment 4: Using Cassiopea xamachana as a bioindicator

Translocation habitat had a highly significant effect on phosphate uptake rates of *Cassiopea xamachana* (Fig. C.4; Table C.1). Translocating jellyfish from the Atlantic side of Key Largo into Florida Bay did not significantly affect their uptake rates compared to reference animals from the original collection site (p=0.67). However, translocating animals from shore and onto coral reefs produced significant increases in uptake rates (see Table C.1 for p-values). Those jellyfish that were moved 8 km from shore to Little Grecian fore reef exhibited the highest

phosphate uptake rates of all treatments while those placed 4.8 km from shore at Admiral patch reef exhibited intermediate uptake rates (Fig. C.4; Table C.1). Water samples from Little Grecian Reef and Admiral Reef revealed undetectable phosphate concentrations (< 0.03 μM DIP; Table C.1) compared to the collection site on the Atlantic side (0.09 μM DIP; Table C.1) and the translocation site in Florida Bay (0.05 μM DIP; Table C.1).

Discussion:

Physiological effects of nutrients on Cassiopea xamachana

Atkinson and Bilger (1992) speculated that changes in the physiology of phosphate uptake by cnidarians, specifically corals, could be a function of past history of nutrient availability. Our results indicate that exposure of *Cassiopea xamachana* to elevated phosphates reduces their subsequent uptake rates and this physiological adaptation can occur in as little as one hour if dissolved phosphates concentrations are high enough. Previous studies show that both zooxanthellae and their associated hosts have reduced uptake rates following exposure to increased environmental phosphates (Deane and O'Brien, 1981; Kelty and Lipschultz, 2002). For example, Belda and Yellowlees (1995) showed that isolated zooxanthellae and host clams (*Tridacna* spp.) respond similarly, with reduced uptake rates occurring after the organisms were exposed to elevated phosphates. These physiological adaptations occur presumably as the organisms become saturated during periods of high nutrient availability. Kelty and Lipschultz (2002) demonstrated that zooxanthellae isolated from a host anemone (*Aiptasia pallida*) for 24 hours were able to absorb phosphate 75 times faster per cell than were freshly isolated zooxanthellae and they attributed this uptake suppression to higher phosphate concentrations measured within host tissue compared to that of filtered Bermudan seawater (Kelty and Lipschultz, 2002). Our findings corroborate these results and suggest that this phenomenon is

widespread among invertebrate-*Symbiodinium* mutualisms. Additionally, we demonstrate that the effects of elevated phosphate exposure on subsequent uptake rates persist for at least one hour and are capable of returning to a typical pre-enrichment pattern in as little as five hours.

It is important to note that phosphate concentrations of 2.0 μM are an order of magnitude greater than those often encountered in coastal and reefs waters (see Szmant, 2002 for discussion). Therefore, after establishing the response observed in the first experiment, we tested whether exposure to low levels of phosphate for lengthier periods would increase the sensitivity of uptake rates in *Cassiopea xamachana*. While animals exposed to the highest phosphate treatment did exhibit significantly lower uptake rates than those incubated at the lowest treatment across all time intervals, increasing the duration of phosphate exposure did not progressively affect uptake rates as predicted. One possible reason for this response may have been that the animals were kept outside and fluctuations in light intensity with the progression of the day may have influenced uptake rates. Previous authors have demonstrated that light affects nutrient uptake rates in anemones and giant clams (D'Elia, 1977; Muller-Parker et al., 1990). This has been attributed to the photosynthetic needs of the endosymbiotic zooxanthellae (Belda and Yellowlees, 1995). In contrast, the animals in the third experiment were kept indoors under continual artificial light and their rates appeared to normalize five hours after exposure and remained constant twenty-four and forty-eight hours later. Our data do not allow us to specifically address the effects of light on phosphate uptake in *C. xamachana*. However, we caution that future work using these animals as comparative bioindicators should be conducted contemporaneously under similar lighting to avoid any confounding influence that light may have on nutrient uptake rates.

Nutrient levels in Floridian nearshore environments

Cassiopea xamachana are capable of integrating past nutrient history into a detectable uptake signature that persists for a period of time. Animals translocated from a common collection site and to Florida Bay and coral reefs revealed comparative differences in environmental phosphates between these sites. *C. xamachana* displayed the lowest uptake rates following environmental exposure at sites with the highest phosphate concentrations as measured by traditional instantaneous time-point sampling. Jellyfish translocated from the collection site on the Atlantic side of Key Largo and into Florida Bay experienced little change in phosphate uptake rates, consistent with the similar dissolved phosphate levels in both habitats. Animals placed on the most distant reef, Little Grecian, had significantly higher uptake rates than those from Admiral Reef despite the fact that phosphates were below detectable levels at both locations using traditional analytical methods. These findings suggest that average ambient phosphate levels were lower at the reef farther from shore. However, it is important to clarify that we did not replicate sites at equal distances from shore, therefore we caution against generalizations about phosphate levels and their relation to distance from shore based on these current data. Also, it is not possible to precisely quantify background phosphate levels using these data, however, inferences can be made about the comparative phosphate levels from one location to another based on the observed relative uptake rates. Future investigations should focus on establishing a phosphate uptake response curve capable of providing more exact approximations of environmental phosphates.

Cassiopea xamachana as a bioindicator

There are several important advantages to using *Cassiopea xamachana* as a bioindicator of dissolved inorganic phosphates. Direct measurement of seawater samples provides information that is inherently time-specific, and therefore may not be indicative of overall

conditions at the tested environments. In contrast, the information obtained by analyzing phosphate uptake rates of *C. xamachana* represents an integration of local phosphate conditions over a lengthier period of time, and thus provides a more accurate portrayal of recent phosphate levels in coastal waters. Furthermore, organismal bioindicators such as *C. xamachana* enable researchers to obtain ecologically relevant information about the physiological effects of phosphate levels on cnidarian-zooxanthellae symbioses in addition to providing comparative information about environmental phosphates. Traditional methods of seawater analysis produce information regarding the ambient phosphate levels at a specific time, but offer no insight as to the ecological impact the nutrient may be having on local animals. *C. xamachana* are particularly useful to researchers because they provide a dynamic model that closely resembles corals. Both *C. xamachana* and hermatypic corals exhibit similar patterns of phosphate uptake related to the nutrient needs of their endosymbionts (D'Elia, 1977). However, *C. xamachana* medusae are not attached benthic organisms, thus making them easily transplantable to different environments for testing purposes. Unlike many corals, *C. xamachana* jellyfish are also readily abundant and are not a protected species. Researchers can therefore examine the effects of increased environmental phosphates on the physiology of a symbiotic cnidarian without contributing to the detriment of threatened coral species. Lastly, using *C. xamachana* allows researchers to make comparisons on the relative concentrations of phosphates in different habitats even though conventional testing methods may not yield distinguishable differences.

Table C.1 Comparative phosphate levels and uptake rates as measured for the four transplant environments.

Site	PO_4 concentration	Mean uptake rates (μmol/gww/h)	P-values for uptake rate comparisons relative to Collectior
Collection site	0.09	0.039438	----------
Florida Bay	0.05	0.043881	0.67
Admiral Reef	Not detectable	0.063707	0.01
Little Grecian Re	Not detectable	0.099076	< 0.001

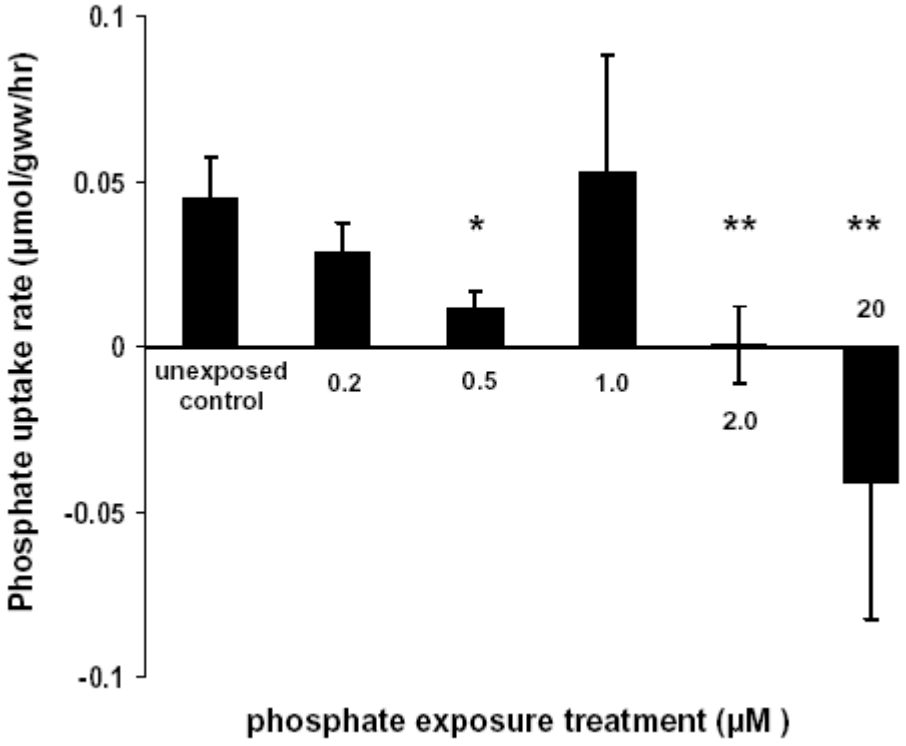


Figure C.1 Mean uptake of dissolved inorganic phosphate ± 1 SE for *Cassiopea xamachana* exposed to elevated phosphates for one hour. Phosphate concentrations for each treatment are listed.

* significant effect of treatment compared to unexposed control group (P <0.05)

** significant effect of treatment compared to all other groups (P <0.05)

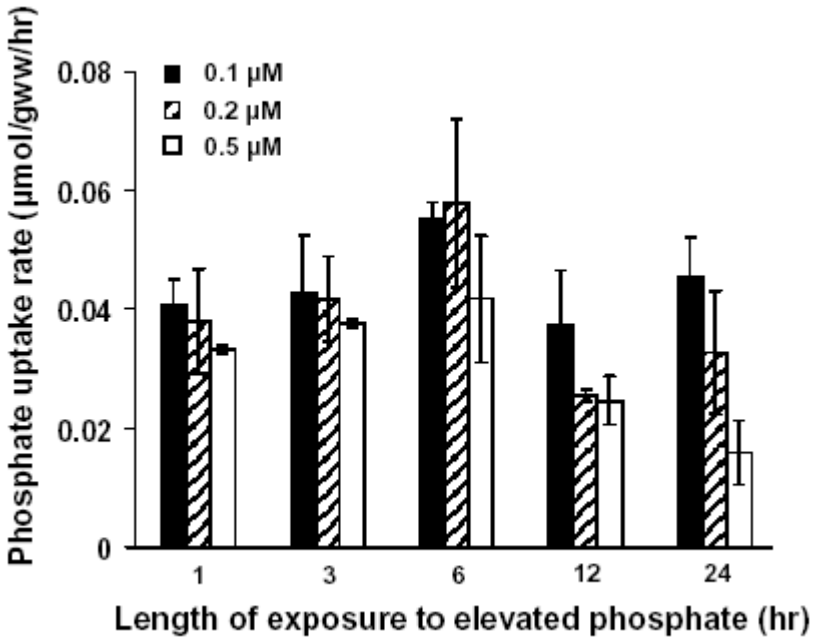


Figure C.2 Mean uptake ± 1 SE of *Cassiopea xamachana* exposed to elevated phosphates. Means represent 3 animals. Exposure to the highest phosphate concentration produced a significant reduction in uptake rates across all lengths of exposure (P <0.05). Uptake rates did not decrease progressively with corresponding increases in length of exposure.

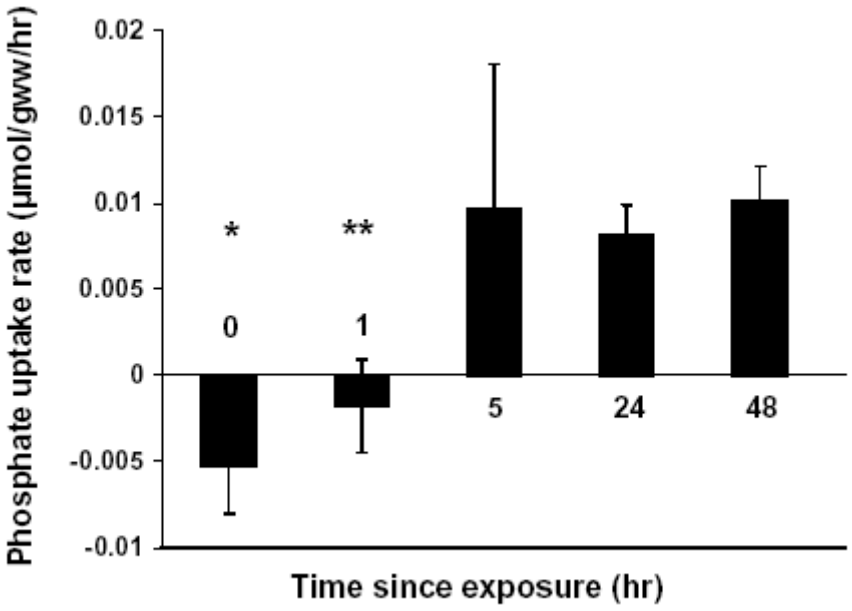


Figure C.3 Mean uptake ± 1 SE of *Cassiopea xamachana* following exposure to 2.0 µM dissolved inorganic phosphate.

* significantly lower uptake rates than all other groups (P <0.05)

** significantly lower uptake rates than groups removed 5, 24, and 48 hours later (P <0.05)

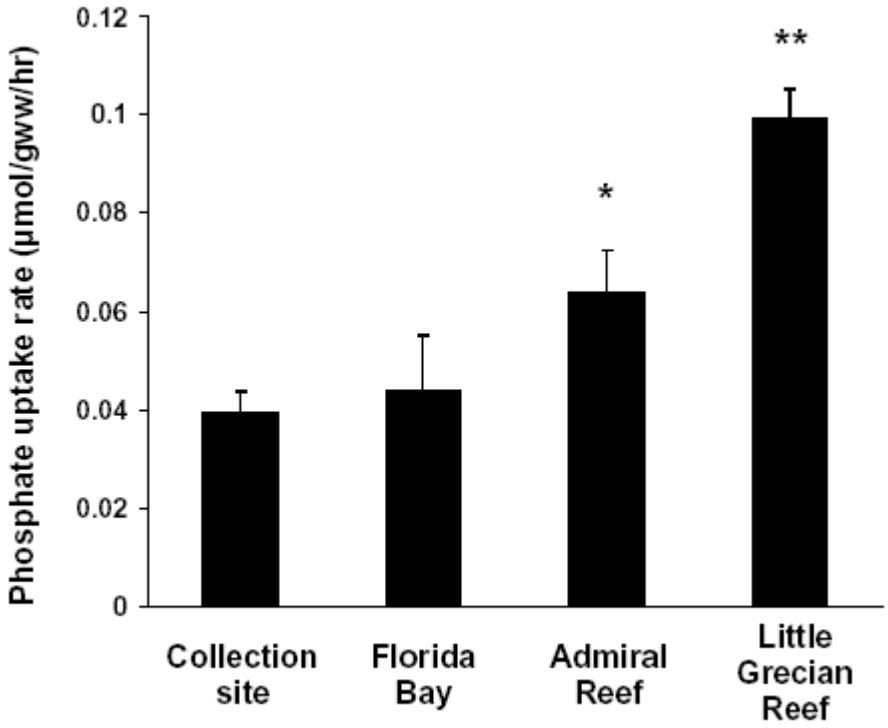


Figure C.4 Mean uptake ± 1 SE for *Cassiopea xamachana* following environmental exposure for four days.

* significant difference between Admiral Reef and Atlantic Canals. (P =0.01)

** significant differences between Little Grecian Reef and all other environments. (P <0.05)

APPENDIX

D – CHRONIC FISH GRAZING IMPEDES CORAL RECOVERY AFTER BLEACHING[9]

[9] Rotjan RD, Dimond JL, Thornhill DJ, Leichter JJ, Helmuth BST, Kemp DW, and SM Lewis. In submission to *Coral Reefs*.

Abstract

Coral bleaching, in which corals become visibly pale and typically lose their endosymbionts (*Symbiodinium* spp.), increasingly threatens coral reefs worldwide. While the proximal environmental triggers of bleaching are reasonably well understood, considerably less is known concerning physiological and ecological factors that might exacerbate coral bleaching or delay recovery. We report a bleaching event in Belize during September 2004 in which *Montastraea* spp. corals that had been grazed by corallivorous parrotfishes showed a persistent reduction in symbiont density compared to intact conspecific colonies. Additionally, grazed corals exhibited greater diversity in the genetic composition of their symbiont communities, changing from uniform ITS2 type C7 *Symbiodinium* prior to bleaching to some mixed assemblages of *Symbiodinium* types post-bleaching. These results suggest that chronic predation coupled with coral bleaching can exacerbate coral stress and destabilize the coral-zooxanthellae symbiosis, and offer a novel explanation for reef-wide patterns of variable bleaching response and recovery.

Introduction:

Coral reef ecosystems worldwide are in decline, associated with escalating stressors such as pollution, eutrophication, and global climate change [1,2]. These anthropogenic stressors have been implicated in coral bleaching events, during which corals become visibly pale typically due to declines in endosymbiont (zooxanthellae) density [3,4]. It is well established that abnormally high seawater temperatures act as proximal triggers of coral bleaching [5] although other factors including high light, pollution, low salinity, and disease have also been implicated [6]. Even though the frequency of coral bleaching has increased dramatically over the past three decades

[3,7], little is known concerning key biological factors that influence coral survival and recovery during bleaching events.

Coral bleaching responses often vary substantially in their extent and severity, even for corals occupying the same physical environment [8-10]. Interspecific variation in bleaching susceptibility has been attributed to physiological or morphological differences within and between coral species [11-13]. *Symbiodinium*, the genus of photosynthetic endosymbionts that associates with corals, is genetically diverse consisting of eight sub-generic clades (A-H), each comprised of numerous "types" [9]. Variability in host bleaching has been attributed to genetic differences in the coral's *Symbiodinium* community [14], which lends support to the hypothesis that coral bleaching may be an adaptive mechanism by which corals obtain stress-tolerant symbionts [15,16]. While these studies indicate that intrinsic differences can contribute to bleaching variability among corals, few studies have examined whether extrinsic biotic factors such as predation can influence coral bleaching susceptibility, intensity, and subsequent recovery.

In the Caribbean, herbivorous fishes (parrotfishes and surgeonfishes) are critical to maintaining healthy coral reefs, as they benefit corals by removing competitively superior macroalgae [17,18]. However, some parrotfish also consume live coral, with direct detrimental effects on coral growth and survival [19-21]. Parrotfish preferentially graze certain coral species [22], as well as particular colonies within a species [21,23]. Parrotfish predation produces characteristic, highly conspicuous grazing scars (Figure D.1a), and often results in partial colony mortality. Although the proximal cause of feeding selectively is not known, parrotfish often repeatedly graze the same coral colonies [21,23-25]. Because corals are clonal organisms, partial predation by parrotfish is likely to be an important source of chronic stress for reef corals.

In this study, we document a coral bleaching event in Belize in September 2004 that occurred in association with elevated seawater temperatures, seasonal fluctuations in zooxanthellae density, and storm surge from Hurricane Ivan. In the context these stressors, we examined the effects of chronic parrotfish grazing on the coral-algal symbiosis. Specifically, we tested whether *Montastraea* spp. coral colonies exposed to chronic parrotfish grazing showed a greater reduction in symbiont density during this bleaching event compared to intact conspecific colonies over time. Finally, we investigated the genetic stability of *Symbiodinium* spp. communities in grazed versus intact coral colonies over the course of this bleaching event.

Results:

Temperature patterns

Coral bleaching was evident in that colonies appeared visibly pale in October compared to August, and re-gained considerable color by January (Figure D.1). The observed coral bleaching coincided with high seawater temperatures at 18m depth, which during August – November 2004, were unusually high compared to the same periods in 2000-2003 (Figure D.2). Most models of coral bleaching concur that bleaching occurs when corals are exposed to water temperatures 1-2°C above the average local maximum for extended periods [3]. The 2m HotSpot temperature threshold for this Belizean reef, reflecting the average local maximum temperature at that depth, has previously been estimated to be 29.85°C [26]. Our data show that in 2004, daily average water temperature measured at 18 m exceeded this threshold for a total of 36 days, including a period of 19 consecutive days in 2004 (September 15-October 3). Moreover, temperatures at 18 m were as much as 0.8°C warmer in September 2004 compared to temperatures recorded during the same times in previous (non-bleaching) years (Figure D.2). Coincident with this period of increased seawater temperatures, the Belizean barrier reef was

affected in mid-September 2004 by storm surge from Hurricane Ivan; we therefore cannot de-couple these events. Hurricane Ivan was a Category 5 storm located approximately 600 km from Carrie Bow Cay at its closest point of 20.4^{o}N, 84.1^{o}W on September 13, 2004, with wind speeds of 140 kt [27].

Zooxanthellae Density

Coral colonies experienced a major decline in zooxanthellae density between August and October 2004 (Figure D.3), resulting in highly significant differences in zooxanthellae density over time (Table D.1). There was also a significant effect of *Montastraea* spp. colony condition (grazed vs. intact) on zooxanthellae density (Table D.1), with intact coral colonies showing overall higher zooxanthellae densities across all timepoints (Figure D.3). In August, prior to the bleaching event, there was no significant difference in zooxanthellae density between grazed versus intact *Montastraea* spp. coral colonies (Table D.1). There was also no difference in October (during bleaching), indicating that grazed and intact *Montastraea* spp. colonies were equally susceptible to bleaching. However, during recovery from bleaching in January 2005, intact *Montastraea* spp. colonies had significantly higher zooxanthellae densities than grazed colonies (Table D.1).

Genetic identity of Symbiodinium *spp.*

Prior to the September bleaching event, we detected exclusively type C7 zooxanthellae in all *Montastraea* spp. corals (N = 50) (Figure D.4). In October, following the declines in zooxanthalle densities (Figure D.3), mixed assemblages of *Symbiodinium* types were detected in several *Montastraea* spp. colonies (N = 6 out of 36 colonies sampled). Furthermore, by recovery

in January, additional colonies showed mixed symbiont assemblages (N = 14 out of 64), but grazed *Montastraea* spp. colonies were significantly more likely to have new *Symbiodinium* spp. types than intact colonies (Table D.2, test of homogeneity of proportions, $\chi^2 = 8.317$, p = 0.0063). For those colonies in which new symbionts were detected, intact colonies always added *Symbiodinium* D1a, whereas grazed colonies added a greater diversity of symbiont types, including types B1, various C types, and D1a.

Discussion

This study documents a coral bleaching event in September 2004 in Belize associated with unusually high temperatures, seasonal fluctuations in zooxanthellae, and storm surge from Hurricane Ivan. Following this event, *Montastraea* spp. colonies subject to parrotfish grazing showed both reduced zooxanthellae density and greater diversity in *Symbiodinium* spp. populations compared to intact coral colonies. Bleaching is an indicator of physiological stress in corals [3,28], and we suggest that parrotfish predation imposes additional physiological stress on *Montastraea* spp. corals that may exacerbate the damaging effects of coral bleaching. Reductions in zooxanthellae density are often associated with catastrophic bleaching, though it has recently been shown that there are also seasonal fluctuations [29,30]. Some studies have documented regular reductions of symbiont density during the warmest times of year in other geographic regions [29,30]. The zooxanthellae reductions and visible bleaching that we observe likely go beyond seasonal lows, intensified by the coincident Hurricane Ivan storm surge and abnormally high seawater temperatures although the bleaching extent did not reach catastrophic levels. Regardless of cause however, our results demonstrate that parrotfish grazing impedes recovery from zooxanthellae reduction.

Although grazing did not appear to influence the susceptibility of colonies to bleaching, it did impede recovery. Mean zooxanthellae density in grazed corals was lower than in intact corals before, during, and after the bleaching event, although this difference only became significant during recovery in January. This is particularly important given that during the winter, corals typically have their highest host tissue mass, symbiont densities and photosynthetic capacity, which ultimately drives coral growth and reproductive output [8,31]. Szmant and Gassman [32] found that the degree of recovery of zooxanthellae populations after a severe bleaching event determined whether or not a coral could complete gametogenesis the following year. In addition to zooxanthellae loss, physical damage also has reproductive consequences for corals: *Montastraea* spp. polyps adjacent to artificially damaged areas were shown to have lower fertility and fecundity compared to undamaged coral tissue [33]. The combination of symbiont loss and physical damage may also interact. Meesters and Bak [34] created artificial lesions on bleached and non-bleached *Montastraea annularis* colonies and found that bleached colonies exhibited less tissue growth, slower lesion recovery, slower tissue color restoration, and higher mortality. Since parrotfish grazing similarly removes coral tissue, grazed colonies are also likely to have slower tissue re-growth following bleaching. These findings, combined with our results, suggest that parrotfish grazing and bleaching may act synergistically to reduce coral fitness.

Environmental change has been previously shown to cause changes in *Symbiodinium* spp. communities [14,35,36]. Prior to the bleaching event, we detected only type C7 *Symbiodinium* in our colonies, as expected since various types of clade C are frequently found in deep-water *Montastraea* spp. throughout the Western Atlantic [37]. After bleaching, we found that a significantly greater proportion of grazed *Montastraea* spp. corals experienced a change in symbiont diversity. Many of the corals that experienced changes in their symbiont community in

our study gained the purportedly stress tolerant *Symbiodinium* type D1a [38,39], which has previously been observed in *Montastraea* spp. colonies following bleaching [35,40]. It is also interesting to note that we detected *Symbiodinium* type B1 only in grazed corals; type B1 is often found in shallow water *Montastraea* spp. colonies and may also be considered somewhat of a generalist due to its symbiosis with a wide variety of cnidarian hosts [37]. In any case, parrotfish predation appears to induce prolonged bleaching, which may increase the likelihood of symbiont change.

Our results suggest that chronic predation coupled with coral bleaching can exacerbate coral stress and destabilize the coral-zooxanthellae symbiosis. Predation stress also offers a novel explanation for reef-wide patterns of variable bleaching response and recovery. It is well established that fish communities are rapidly changing, [41,42], coral cover is declining [1,2], and the world's oceans are getting warmer [7]. As a result, it is crucial to understand the synergistic effects of multiple stressors, including chronic fish grazing, on coral survival and recovery.

Methods:

Study site & sampling

This study was conducted at Carrie Bow Cay, Belize (16º 48' N and 88º 05' W) on the outer ridge of the Belize barrier reef (~ 18m depth). *Montastraea faveolata* and *M. franksi* corals are the major reef building corals in this habitat, and approximately 33% of these colonies experience grazing by parrotfish (data not shown). We sampled marked colonies of grazed and intact *M. faveolata* and *M. franksi* corals at 3 time periods: in August 2004 (N = 64 colonies), October 2004 (N = 22) and in mid-January 2005 (N = 24). August and October 2004 sampling

periods bracketed a period of considerable environmental disturbance characterized by unseasonably high temperatures concurrent with physical destruction caused by storm surge from Hurricane Ivan, in addition to seasonal lows in zooxanthellae densities [described by 8,43]. The January/February 2005 sampling occurred during the coolest months of the year, when corals generally exhibit the fastest rates of skeletal growth and increase in tissue mass [8,44]. We classified coral colonies as grazed if they showed at least 6 distinct grazing scars; most grazed colonies had more than 30 distinct bites. Intact colonies had no grazing scars. Small samples (< 5cm^2) were collected by SCUBA with a hammer and chisel. When sampling grazed colonies, care was taken to remove tissue only from areas adjacent to grazing scars rather than from the scars themselves.

Temperature analysis

Ambient water temperatures were recorded from March 2000 through February 2005 using a combination of Onset Computer Corporation Stowaway, Tidbit, and Watertemp Pro loggers with 0.2 °C resolution and a 1 – 5 min response time. These instruments sampled temperature every 0.5 s and recorded 10-16 min averages (except Watertemp Pro loggers which recorded 10-16 min interval points). Instruments were calibrated against a Seabird Electronic SBE 39 logger (0.001 ^{o}C resolution, 20 s response time) in the laboratory and in the field, and were found to be within factory specifications. Daily average water temperatures for 2004 were compared to day-of-the-year averages calculated across the preceding 4 years.

Zooxanthellae Density

To determine zooxanthellae density for *Montastraea* colonies, we completely removed approximately 5 cm^2 of coral tissue from colony samples using a WaterPik® with filtered seawater [45]. Tissue was homogenized with a blender and zooxanthellae were counted at 100X magnification using a hemacytometer (10 replicate subsamples). The total volume of the tissue homogenate was recorded and coral surface area was measured using aluminum foil [46] in order to calculate zooxanthellae density per cm^2 of coral tissue. A 2-way ANOVA was used to examine condition (grazed vs. intact), time (month), and interaction effects. To determine specific differences between grazed and intact colonies during each sampling period, we used linear contrast t-tests for pre-planned multiple comparisons (SAS).

Establishing Symbiodinium *spp. ITS2 type*

To determine the genetic identity of *Symbiodinium* spp. populations associated with the coral colonies, zooxanthellae (algal cells) were isolated from the remaining coral tissue by centrifugation at ~6700 g's for 3-4 minutes. Algal pellets preserved in 70% ethanol were transported back to the U.S., and nucleic acids were extracted using the Wizard DNA preparation protocol (Promega) following the methods of LaJeunesse et al. [47]. The internal transcribed spacer 2 region (ITS 2) of nuclear ribosomal RNA was used to discriminate molecular types of *Symbiodinium* [37,48]. This region was amplified from the DNA extract for denaturing-gradient gel electrophoresis (DGGE) using primers "ITS 2 clamp" (5'CGCCCGCCGC GCCCCGCGCC CGTCCCGCCG CCCCCGCCC GGGATCCATA TGCTTAAGTT CAGCGGT-3') and "ITSintfor 2" (5'GAATTGCAGA ACTCCGTG-3'). PCR amplification followed the "touchdown" thermal cycle protocol of LaJeunesse [37]. Products of these PCR reactions were

checked by electrophoresis on agarose gels (0.8% agarose in 40 mM Tris-acetate, 1mM EDTA solution). Successfully amplified PCR products were subsequently electrophoresed on denaturing gradient gels (45-80% formamide, 8% acrylamide denaturing gradient gels; 100% consists of 7 *M* urea and 40% deionized formamide) following the protocol of LaJeunesse and Trench [49], with the modifications of LaJeunesse et al. [47].

Table D.1 Statistical analysis of changes in zooxanthellae density in *Montastraea* spp. corals in Belize. Two-way ANOVA shows effects of coral condition (grazed versus intact), sampling time, and interaction. Grazed versus intact colonies were compared at each sampling month using planned linear contrasts.

Source	**SS**	**df**	**F**	**p**
Condition (G vs. I)	11.809	1	5.43	0.0217
Time (Aug, Oct, Jan)	43.636	2	10.03	0.0001
Condition * Time	4.544	2	2.27	0.3556
August (G vs. I) ***Pre-bleaching***	1.193	1	0.55	0.4607
October (G vs. I) ***During-bleaching***	2.220	1	1.02	0.3148
January (G vs. I) ***Post-bleaching recovery***	9.967	1	4.58	0.0347
Error	228.48	105		
Total	57.76	5	5.31	0.0002

Table D.2 Number and percentage of *Montastraea* colonies exhibiting a change in *Symbiodinium* communities from August (pre-bleaching) to January (post- bleaching recovery). The same colonies were sampled at each time point.

	Clade change	*No change*	**% change**
Grazed	10	20	33%
Intact	4	28	13%

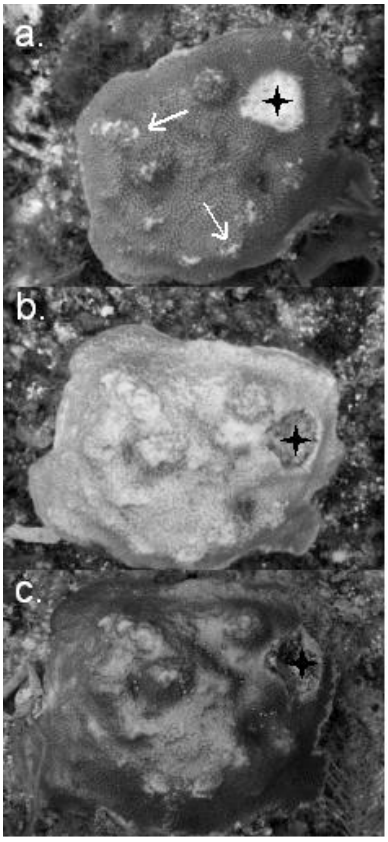


Figure D.1 Time series of a single grazed *Montastraea* spp. coral colony from (a) pre-bleaching in August 2004 to (b) October during bleaching to (c) recovery in January at Carrie Bow Cay, Belize. Relative bleaching levels can be visualized by contrasting the varying appearance of the colony to the reef floor. Arrows in (a) indicate representative grazing scars; the star in all 3 photos represents a sampling scar on the colony.

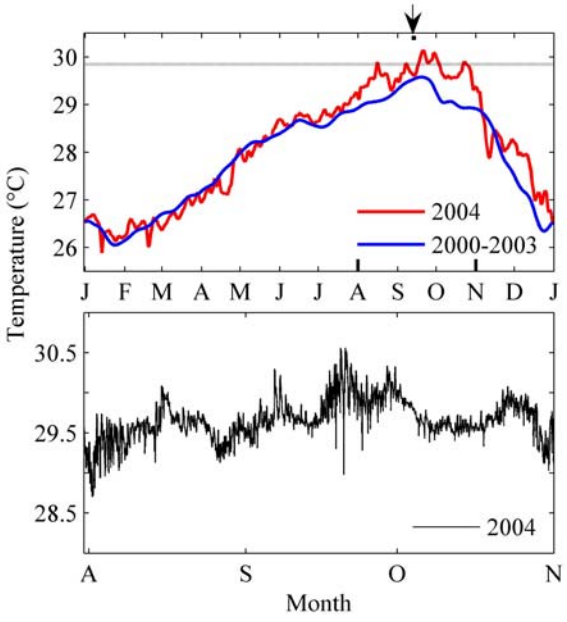


Figure D.2 Changes in water temperature over time at 18m depth on the Belize Barrier Reef, Carrie Bow Cay (CBC), Belize. Top panel shows the average daily water temperatures for 2004 compared to a 7-day running mean of the day-of-the-year average for the previous 4 years (2000-2003). Dashed line represents the Pathfinder HotSpot threshold, 29.85^{o}C, for 2m depth at Carrie Bow Cay established by Aronson et al. (2002). Bottom panel shows a close-up of 10-min interval temperature data around the time of the bleaching event, August – November 2004. Arrow at the top indicates when storm surge from Hurricane Ivan first affected CBC (12 – 15 Sept 2004). Bold ticks along the bottom of the top panel indicate the period of the close-up shown in the lower panel.

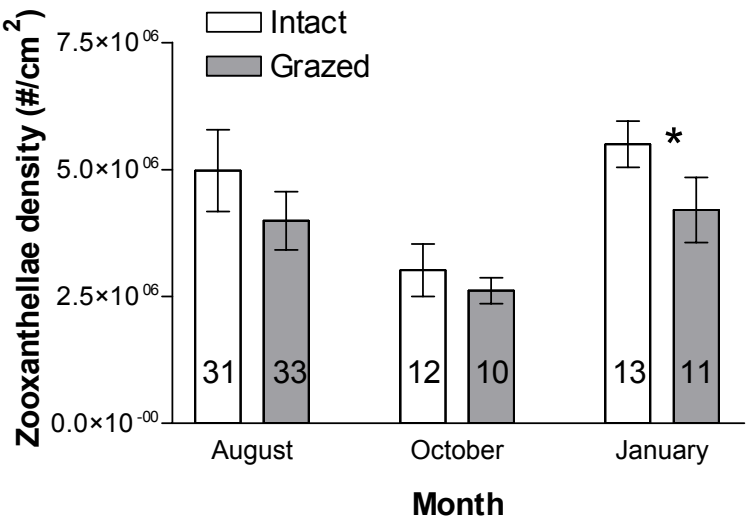


Figure D.3 Zooxanthellae densities within parrotfish-grazed vs. intact *Montastraea spp*. colonies in 2004-5 prior to the bleaching event (August), immediately following the bleaching event (October), and during recovery from bleaching (January). Sample sizes are represented within each bar; bars represent mean ± S.E. Linear contrasts were used to compare grazed and intact colonies for each month; * indicates $p < 0.05$.

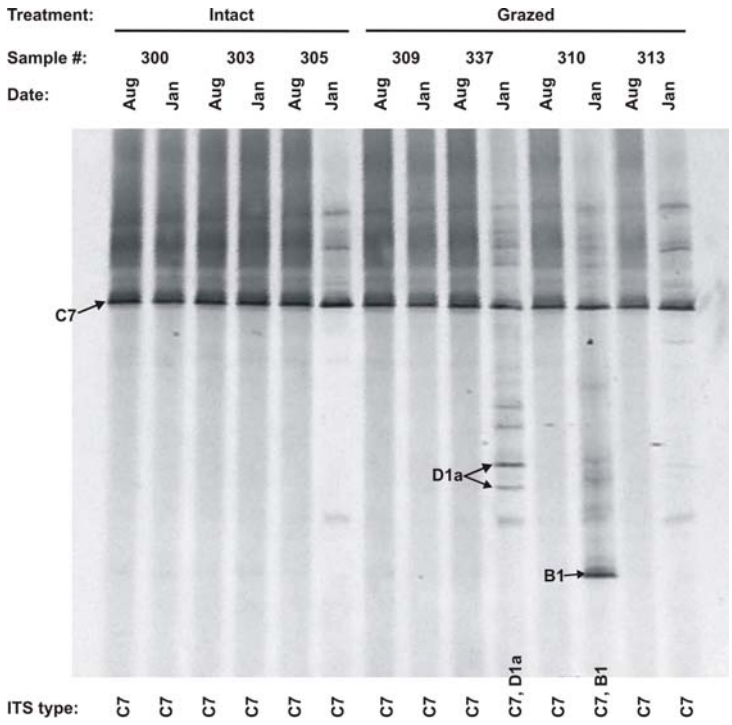


Figure D.4 PCR-DGGE profile of the *Symbiodinium* ITS 2 region showing symbiont types detected in representative grazed vs. intact *Montastraea* spp. colonies (N = 64) sampled prior to bleaching (August 2004) and during bleaching recovery (January 2005). Profile is presented as a reverse image. Diagnostic bands are labeled for types B1, C7, and D1a.

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