The Stability of the Giant Clam Holobiont over Time and during Bleaching Stress

Thesis by
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ABSTRACT

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The stability of marine photosymbiotic holobionts has major implications for the future of coral reef communities. This study aims to describe the stability of the Red Sea giant clam holobiont over the duration of one year and during induced bleaching stress under laboratory thermal manipulations. Tridacnid clams of the species *Tridacna maxima* were sampled at three reef locations near the central Saudi coast of the Red Sea. Associated *Symbiodinium* of Red Sea giant clams have previously been described to be part of only Clade A, which suggests a strong specificity in the clam-algal partnership, but specific types and potential shifting of types within this clade have not been examined for giant clams. The results from this study confirm that tridacnid symbiont types shift over time and the change between three A1 types suggests a biological and functional significance of two undescribed A1 *Symbiodinium* types.

Experimental bleaching shows that Red Sea giant clams, although exposed to rather hot temperatures naturally, will bleach at 34°C after two weeks, and severely bleached clams likely will not recover. During bleaching, *Symbiodinium* types shift as well, and shift more drastically than seasonal shifts during the year. This shifting may be an evolved characteristic of the giant clam to aid in surviving major changes in the environment. However, more research is needed to determine if these holobionts are capable of keeping up with the global forecast of warming in reef environments.
ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

ABH: Adaptive bleaching hypothesis
BCL: Biosciences core lab
BT: Bleaching threshold
CMRCL: Coastal and Marine Resources Lab facilities at KAUST
DHW: Degree heating weeks
IUCN: International Union for Conservation of Nature
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1. INTRODUCTION

The relationship between zooxanthellae and animal hosts began about 210 million years ago in the late Triassic period (Stanley & Swart, 1995; Stanley, 2003). Zooxanthellae, a type of endosymbiotic dinoflagellate algae, allows corals to survive...
and grow in nutrient-poor waters by providing photosynthate and oxygen to the animal and support calcification of the coral skeleton (Stanley, 2006). The importance of this relationship is emphasized by its endurance throughout time, but the relationship is proving to be quite fragile in the current changing climate (Frankowiak et al., 2016).

In corals, the zooxanthellae exist in a single-cell layer in the gastrodermis tissue of the host. The translucence of the coral epidermis allows light to reach the algae. The excess photosynthate and oxygen produced by the algae is used by the coral, while the coral provides a safe niche for the algae (Muller-Parker et al., 2015). The relationship between these two organisms is essential. However, corals are not the only marine invertebrates with this relationship. There is a wide range of invertebrates that host zooxanthellae including sponges (Carlos et al., 1999), nudibranchs (Kempf, 1984), anemones (LaJeunesse & Trench, 2000), and giant clams (Muscatine, 1967; Carlos et al., 1999; Ishikura et al., 2004; Stat et al., 2006).

Giant clams, or tridacnids, are found in the tropics of the Indo-Pacific, the Great Barrier Reef, and the Red Sea (Othman et al., 2010). They have formed a symbiotic relationship with algae, which has influenced their morphology, growth, and life history (Fitt et al., 1986). The gigantic size of these bivalves is only possible due to this relationship (Yong, 1975), but their size is not their only differentiating trait among the bivalves. A 180-degree rotation of the shell orientation separates tridacnids from their ancestral cockles (Yong, 1975). Also, the enlarged mantle tissue, which harbors the zooxanthellae, protrudes from their shells, opening upward toward the sun (Hernawan, 2008). Some giant clams cannot close
completely due to the mantle enlargement and their adaptive behavior of remaining open. The eleven species of giant clams, two in the genus *Hippopus* and nine in the genus *Tridacna* (Borsa et al., 2015), have evolved to accommodate their algal symbionts in this way (Yong, 1975). It should be noted that tridacnids store their algal symbionts differently from most corals. Zooxanthellae cells are stacked in tubules that extend from the stomach cavity of the bivalve to the mantle tissue (Mansour, 1946). Located in the mantle tissue are specialized cells called iridophores that contain protective pigments which block harmful light wavelengths and act as mirrors, reflecting light to stacked algae cells (Holt et al., 2014). In most corals, the algae form a single-cell layer in the gastrodermis tissue of the animal (Douglas, 2003). Another difference among corals and clams in terms of symbiosis is the clam’s higher dependence on heterotrophic filter feeding. While corals gain most of their energy requirements from their zooxanthellae, clams maintain an important filter feeding behavior that contributes to at least one third of their required carbon intake (Klumpp et al., 1992). Knop (1996) argues that despite photosynthetic symbionts, the presence of a fully-developed intestinal tract implies a degree of dependence on filter feeding to survive, and giant clam aquarists intentionally supplement phytoplankton to enhance clam survival and growth. It has been found that, as juveniles, tridacnids rely heavily on filter feeding to acquire their food source, but as they grow larger, the photosymbiosis with their associated *Symbiodinium* becomes more important once they have established a stable relationship with preferred zooxanthellae (Klump et al., 1992; Klump and Griffiths, 1994). Some species of tridacnids have greater heterotrophic capacity than others,
and therefore may have greater tolerances to bleaching (Jantzen et al., 2008). The ability to feed both heterotrophically and autotrophically gives mixotrophs an advantage in low-nutrition environments or in times of environmental change, such as warming temperatures. This symbiotic relationship is outstanding and has allowed for these unique creatures to exist. The beauty of the giant clam holobiont is only a byproduct of the truly mutualistic and essential relationship. Although the clam can obtain food by filter feeding, it does not account for the entire amount required for clam growth and stability, therefore giant clam bleaching is a relevant threat.

The endosymbiotic algae or zooxanthellae, literally meaning “yellow animal” in ancient Greek, are single-celled dinoflagellates classified in the genus *Symbiodinium* (Muller-Parker et al., 2015). Although the genus *Symbiodinium* covers the known species in photosymbiotic holobionts, zooxanthellae still serves as an accurate term due to the ambiguity of species delineation and diversity within the realm of symbiotic algae. Therefore, the terms *Symbiodinium* and zooxanthellae will be used interchangeably in this paper. Currently, the genus *Symbiodinium* includes nine identified clades labeled A to I (Pochon & Gates, 2010). However, the clade system has been criticized for its inability to capture the immense diversity of these endosymbiotic algae and the organization of all clades under the same genus is also being questioned (Rowan & Powers, 1992; Stat et al., 2008; Pochon & Gates, 2010). Within one clade, there are many diverse types with genetic divergences matching the level of differences seen across entire orders of other dinoflagellates (Rowan & Powers, 1992). This level of diversity has been shown for Clade D *Symbiodinium*,
where just a few nucleotide base changes indicate a significantly different "species" with different ecological functions (LaJeunesse et al., 2010; LaJeunesse et al., 2014). Research investigating certain clades and types of *Symbiodinium* has shown that specific types within Clade C have very different tolerances to thermal stress (Sampayo et al., 2007; Hume et al., 2015), further supporting the fact that cladal categorization, when describing ecological function, proves to be too broad and thus, inaccurate. Clade D and C have been found to include types with high diversity, indicating species-level differences, but proper species differentiation is not comprehensive, thus, these different OTUs are referred to as types or strains (LaJeunesse et al., 2004; Lajeunesse et al., 2014). The high diversity within these clades suggests potentially high diversity within other clades as well. For example, Clade A, a common clade found in corals and tridacnid clams in the Red Sea (Pappas et al., 2017; Ziegler et al., 2017), has been classified as an opportunistic, stress tolerant, and sometimes parasitic clade (Stat et al., 2008; Lesser et al., 2013). However, there are 18 types in Clade A with more being identified (see LaJeunesse et al., 2015), some of which do not fall into these characteristic categories, suggesting a much wider ecological diversity than originally perceived. With more research being done on the diversity of the genus *Symbiodinium*, it is clear that characteristics of entire clades are not accurate (Sampayo et al., 2007). Thus, types within a clade may have very different ecological advantages, and harboring different types could allow for different functioning of the holobiont.

The research perspective of examining these organisms as a system that responds to stress dynamically is growing. Photosymbiotic organisms such as corals
and giant clams are considered holobionts, which refers to the entirety of the organisms involved in the symbiotic relationship. The holobiont is not a foreign concept; we are familiar with the human body as a holobiont due to our gut microbiome and other bacteria that keep us healthy (Rosenburg & Zilber-Rosenburg, 2011; Costello et al., 2012; Singh et al., 2013). In the case of corals and giant clams, the holobiont includes the animal host, the symbiotic algae, symbiotic bacteria, and associated viruses (Bourne et al., 2009). This concept acknowledges that the entire holobiont responds to changes in its environment and acts as a unit which evolves together (Rosenburg & Zilber-Rosenburg, 2011).

A major threat to these holobionts is climate change. Increasing sea surface temperatures (SST) are exceeding the thermal limits of many of these organisms, weakening the symbiotic relationship. High temperatures negatively affect the photosynthetic ability of the zooxanthellae, causing their death or expulsion from the animal host (Warner et al., 1999; Tchernov et al., 2004). In either case, the animal loses its major food and energy source. Due to the pigmentation of the zooxanthellae, when the zooxanthellae are expelled from the animal host, the animal loses most of its color in the living tissue, exposing the white color of the underlying skeleton. Thus, the phenomenon of coral bleaching describes the visual observations of stress. An increase in SST for long periods of time can cause bleaching events, but, changes in irradiance levels, salinity, turbidity, and other stresses can cause bleaching as well on local to regional scales (reviewed in Glynn, 1993; Brown, 1997; Douglas, 2003). Although there are many potential causes or triggers of a bleaching event, increasing SST has been the only one correlated to bleaching on a global scale.
Furthermore, bleaching events are predicted to increase in frequency and intensity in the next 50 years due to the increasing temperatures (Wilkinson, 1999; Hoegh-Guldberg, 1999; Hoegh-Guldberg et al., 2007), and we have already seen mass bleaching events that have devastated reefs worldwide due to high SST (see Ku'ulei et al., 2017; Hughes et al., 2017; Hoegh-Guldberg et al., 2017).

To discuss the phenomenon of bleaching with standard measurements, the National Oceanic and Atmospheric Administration’s (NOAA) Coral Reef Watch Program has established important indices used for describing bleaching intensity. Those include coral bleaching "HotSpot" anomalies, “Bleaching Threshold” (BT), and “Degree Heating Week” (DHW) (Liu et al., 2003). To calculate these indices, NOAA provides a maximum monthly mean (MMM), which is the temperature that represents the hottest month over the course of >50 years for a given region. A HotSpot is identified whenever the SST of that region exceeds this historically determined MMM. Bleaching in corals has been observed to occur when the SST reaches 1°C above the MMM. The temperature that equals 1°C above the MMM is considered the Bleaching Threshold. HotSpot anomalies indicate the occurrence of the hottest temperatures in the hottest months of a region, identifying the intensity of the thermal stress (Liu et al., 2003). However, HotSpot anomalies are not enough to predict the thermal effects on biological systems. Thus, DHW represents the accumulation of HotSpots that have exceeded the BT in a given region over a 12-week time period (Liu et al., 2003). For example, a 1°C-heating week indicates that at that given time, the corals have experienced an accumulated thermal stress of 1°C.
above the MMM for a duration of three months (Liu et al., 2003). DHW is a consistent way to measure thermal stress on photosymbiotic holobionts. Universal measurements and consistency can aid in better prediction of global bleaching events, and by studying tolerances, adaptability, and limits of the affected organisms, we can be better prepared for the future of our reefs and marine resources. Therefore, in bleaching experiments, it is ideal to calculate DHW at which bleaching occurs.

The holobiont’s ability to shift *Symbiodinium* types and the potential benefit of that in terms of bleaching has been a recent focus in holobiont research (Baker et al., 2004). The Adaptive Bleaching Hypothesis (ABH), a term coined by Buddemeier and Fautin (1993), suggests that corals expel certain types of *Symbiodinium* and keep or take from the environment fitter types. Most corals will naturally harbor more than one type of *Symbiodinium* (this includes types in the same clade and types of different clades) with one type dominating in abundance (Rowan et al., 1997; Baker, 1999; 2001; Glynn et al., 2001; Pawlowski et al., 2001; Pochon et al., 2001; Santos et al., 2001; Van Oppen, 2001; LaJeunesse 2001; 2002; LaJeunesse et al., 2003). In times of stress, background types may shift to the dominant position if better suited for that role (Rowan & Knowlton, 1995; Baker & Romanski, 2007; Jones et al., 2008). Corals in particular have been the main focus for the research on this symbiont shuffling. Research from the Great Barrier Reef, the Caribbean, the Red Sea, and the Indo-Pacific has suggested that corals can actively shift clades or types of *Symbiodinium* during bleaching stress (Rowan and Knowlton 1995; Rowan et al. 1997; Glynn et al. 2001; LaJeunesse et al. 2004; van Oppen et al. 2005; Stat and
Gates 2010). This shift suggests that certain clades or types within a clade are better suited for increases in heat, irradiance, or stress. Clade D has been suggested to be a heat tolerant clade (Stat et al., 2010; Oliver and Palumbi, 2011; Ladner et al., 2012; DeBoer et al., 2012), and some corals that harbor Clade C have been shown to shift to a dominance in Clade D types during times of bleaching stress (Jones et al., 2008; Jones and Berkelmans, 2011; Kemp et al., 2014; Davies et al., 2017). However, a specific type in Clade C, *Symbiodinium thermophile*, has been found to provide thermal tolerance to corals in the Persian Gulf, one of the hottest seas hosting coral reefs (Hume et al., 2015). The thermal tolerance discrepancies amongst clades suggests that functional characteristics on a cladal level may not be as simple as originally thought. Again, due to many environmental factors, host species, and geography, it is even harder to identify specific ecological roles of *Symbiodinium* types. For example, types found in one animal host may provide thermal tolerance, while in another host decrease the host’s ability to resist bleaching (see Sison, 2003; Venn et al., 2008). Furthermore, types thought to be thermally tolerant may simply just be opportunistic and become dominant due to the lack of competition (Baker, 2001; Douglas, 2003). Much more research on the dynamics of *Symbiodinium* shuffling, the diversity of the many types within the genus, and the shuffling process in other animal hosts is needed to understand the extent to which this technique can aid in the adaptability of photosymbiotic animals.

The research on *Symbiodinium* shuffling in hermatypic corals is of high priority due to the importance of coral reef ecosystems. However, there is a lack of research on other photosymbiotic holobionts, like the giant clams, and how they
potentially shuffle *Symbiodinium* types as well. The evidence of shifting symbionts in corals suggests shifting of symbionts in giant clams could be possible. Previous studies on *Symbiodinium* types harbored by giant clams show association with Clades A, C, and D (Baillie et al., 2000; DeBoer et al., 2012). Ikeda et al. (2017) showed that different species of giant clams host different clades of *Symbiodinium*, and suggests that irradiance, water temperature, and size might be the root of these differences (Ikeda et al., 2017). It has also been shown that juvenile tridacnids actively change their *Symbiodinium* types before establishing a more stable relationship with one or a few preferred types (Belda-Baillie et al., 1999). Because corals have been shown to shift their symbionts with environmental changes and giant clams host multiple types of *Symbiodinium*, it is plausible that giant clams will shift these types as well. However, there are many differences between the coral holobiont and the clam holobiont that may result in differences in the symbiotic relationship dynamics. For example, clams store their zooxanthellae differently than corals, which may have an effect on the expulsion of zooxanthellae during thermal stress. Also, clams have a higher heterotrophic capacity, which may result in the stronger ability to survive through bleaching events. These and other differences may then have implications in symbiont shuffling and bleaching tolerances among corals and clams.

Research on the shifting potential of giant clam symbionts is important in itself, but is also important for understanding the process and extent to which shifting occurs in photosymbiotic holobionts overall. Research on the giant clam’s ability to shift *Symbiodinium* types may support findings from coral studies and lead
an identification of a type of *Symbiodinium* that can withstand future temperatures. Research on the giant clam holobiont will provide a better understanding of their adaptability to climate change, and, due to their threatened status (as listed by the IUCN), the importance of this research that could better conservation efforts is emphasized.

This thesis aims to examine the giant clam's symbiont community over time (seasonally) and during experimentally-induced bleaching. Tridacnid clams in the Red Sea primarily harbor Clade A *Symbiodinium* (Pappas et al., 2017), but classification of types has not been examined to its full extent. With the technology of Next Generation Sequencing (NGS) through the Illumina Miseq platform, specific types and proportions of each can be identified in individuals across time. Evidence of symbiont shifting has not been recorded for giant clams, but has the potential to support the Adaptive Bleaching Hypothesis for other photosymbiotic invertebrates.

Studying these organisms in the Red Sea also has significance for climate change research. The Red Sea is geologically quite young but experiences some of the hottest and most saline conditions in the world of coral reefs (Berumen et al., 2013). The Red Sea is home to a diverse range of organisms already evolved for the harsh conditions of climate change predicted for reef systems around the world. Thus, research on symbiont shifting and stability in Red Sea tridacnids could tell us the future of adaptability in giant clams and other holobionts globally. This creative approach in examining holobiont symbiosis will offer novel observations with implications for the future of tridacnid symbiosis and holobiont adaptability to a warming ocean.
This study includes both field work with sampling *in situ*, which is referred to as the seasonal tridacnid sampling, and experimental work with samples taken from bleached tridacnids in the laboratory, which is referred to as the experimental bleaching sampling. The methods and results are presented separately for these two approaches, but results are discussed together.

The examination of the giant clam as a stable holobiont starts with determining its stability over time *in situ*. To determine this baseline holobiont, clams were marked and sampled over the period of one year. With no thermal stress outside of the normal temperature fluctuations, the community of *Symbiodinium* of each individual should represent a normal giant clam holobiont. As many corals have been found to remain stable over time when extra stresses are absent (Baker, 2003; Thornhil et al., 2005; Goulet, 2006), I expect the giant clam holobiont to remain stable over time during this sampling year. As studied previously, all clams sampled in this region harbor type A1 or *Symbiodinium microadriaticum*, and I expect this dominant type to remain dominant over the four seasons with change only present in background type proportions. However, due to the numerous accounts of symbiont shuffling observed in corals during thermal stress (Jones et al., 2008; Jones and Berkelmans, 2011; Kemp et al., 2014; Davies et al., 2017), I expect a shift in the dominant types of *Symbiodinium* in clams exposed to high, bleaching-inducing temperatures in the bleaching experiment. I expect a shift not only in the dominant type, but also in background types for clams under bleaching stress. Genetic data will show dominant types based on the ITS gene region of the *Symbiodinium* with Sanger sequencing. For further and more detailed analysis, NGS
and the use of the SymPortal program (Hume et al., unpublished) will show the potential shifts in the proportions of all types present within a sample. With this data, the potential shift in symbiont community can be observed for giant clams, and a pattern may emerge in bleached vs. healthy clams.

The studies of diversity within *Symbiodinium* have commonly used the ITS and ITS2 region of the genome to examine sequence divergences amongst different types (Lajeunesse, 2001; 2002; Goulet, 2006; Jones et al., 2008; Hume et al., 2015; Davies et al., 2017). In eukaryotes (thus *Symbiodinium*) there are two ITS regions. ITS1 is located between two rRNA genes, 18S and 5.8S, and ITS2 is located between 5.8s and 26S (in plants, or 28S in animals) (Hunter et al., 2007). The location of these regions is important in that they function as a buffer for mutations as the entire region undergoes amplification. The multiple copies produced during the amplification of the region allows for easy detection of the ITS regions within a sample. Having a high abundance in the genome of the organism, the ITS regions are ideal for genetic analysis. Additionally, due to the multicopy nature of the ITS regions and their function as buffers for mutation, mutations that occur in these regions allow for identification of different species even if those species are closely related (Song et al., 2012). For these reasons, the ITS regions are commonly used to delineate species or types within the genus *Symbiodinium*. The genetic work in this study utilizes the ITS region, as it has been referred to as a universal barcode for dinoflagellates (Yao et al., 2010; Stern et al., 2012).

Bleaching has been observed in corals when temperature increases by 1°C above the maximum monthly mean for the duration of one month (Donner, 2011).
Red Sea corals already live at bleaching-inducing temperatures of other regions and thus, have a much higher bleaching threshold. Studies from the Gulf of Aqaba have shown that corals do not bleach until temperatures reach 33°C for one month (Fine et al., 2013; Grottoli et al., 2017). Due to such high thermal tolerances, bleaching in the Red Sea has not been as severe as bleaching elsewhere, and temperatures above the bleaching threshold had not even been recorded in the Red Sea until after 1995 (Chaidez et al., 2017). However, the Red Sea has recently been affected by the major global bleaching event in 2015 with temperatures reaching 33°C across large areas for multiple weeks (Chaidez et al., 2017). With recorded maximum temperatures of inshore reefs exceeding 35°C (Davis et al., 2011) and a fast-warming pattern of the Red Sea (Chaidez et al., 2017), the coral reefs here are likely close to reaching their thermal limits. Clams and corals may have different tolerances to bleaching, however. I expect clams to have a greater tolerance to bleaching than corals due to the clam’s greater heterotrophic capacity, biomass, and lipid storage, which have been characteristics responsible for some coral species to be tolerant of bleaching (Grottoli et al., 2017). Also, I personally observed in November 2016 in the Red Sea an abundance of healthy tridacnids among bleached corals, indicating that clams had survived through the 2015 bleaching event and thus have a higher thermal tolerance. I expect clams to bleach at temperatures >33°C, the temperature at which large-scale bleaching occurred in Red Sea corals in 2015. An objective for this bleaching experiment is to determine the bleaching threshold for tridacnids located on inshore reefs of the central Saudi coast of the Red Sea. Bleaching due to stresses other than temperature fluctuations, such as handing or aquaria-related stress, will
need to be controlled for so as to only monitor bleaching due to increased
temperature. The results from the experimentally bleached samples will indicate the
clam’s thermal tolerance and can help us better predict their future population
health in the face of a quickly warming Red Sea.
2. MATERIALS & METHODS

2.1: Seasonal Tridacnid Sampling Methods

2.1.1 Project design

To determine the stability of the giant clam as a holobiont throughout time, 10 individuals at each of three different reefs on the central Saudi coast of the Red Sea were identified, tagged, and sampled for one year. General characteristics of these reefs are described in Khalil et al. (2017).

2.1.2 Tagging

The three sampling reefs were chosen based on distance from the coast and were categorized as inshore, mid shelf, or offshore reefs, following a design similar to my previous project on giant clams in this region (Pappas et al., 2017) (Fig. 1). Results from the same study determined that there are two species of giant clams present in the sampling region, *Tridacna maxima* and *Tridacna squamosa* (Pappas et al., 2017). To eliminate potential interspecific variation in holobiont properties, species identification was done in situ, and only *Tridacna maxima* was used as the study species. All individuals were located between 5 and 10 meters depth and were roughly the same size (approx. 14 - 22 cm). Tissue collection and tagging were done via SCUBA. Floats were attached to the reef adjacent to each individual (Fig. 1c) and were removed upon completion of the project. GPS coordinates were recorded for each sampling site.

2.1.3 Sample collection
Collection dates were as follows: November 2016, February 2017, May 2017, and August 2017. The four collection times were chosen to determine the *Symbiodinium* type community of the tridacnid holobiont over time. Tagging was done on the November 2016 sampling date. A photo of each individual was taken and depth was recorded. The sampling procedure of each individual started with placing a metal rod (approximately 1.5 cm in diameter) lengthwise between the shells of the tridacnid to keep the shells open enough for sampling. Then, using forceps, the edge of the mantle tissue was grasped and a small piece (2-3 cm²) was cut with scissors and placed in 1.5 ml tubes labeled with date, individual number, and reef. On the boat, the tissue samples were then rinsed with Milli-Q water and each was cut in half. Half of the sample was stored in 96% ethanol and the other was frozen in liquid nitrogen until transferred to the -80°C freezer. Some marked individuals did not survive the entire year of sampling (Table 1), and when this occurred, other clams of similar size and depth at the sampling site were collected to keep the sample size for seasonal sampling consistent.
Figure 1. (A) Map of the region of Saudi Arabia and the Red Sea. (B) Specific reefs sampled in this study near Thuwal. (C) Photo depicting the general tagging of individual clams in situ. (D) Photo of marked tridacnids in situ.
Table 1. Mortalities of marked individual tridacnids on each reef sampled throughout the year. Numbers represent mortalities that occurred before listed sampling date.

<table>
<thead>
<tr>
<th>Sampling Date</th>
<th>Shib Nazar</th>
<th>Shark Reef</th>
<th>Abu Shosha</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nov. 2016</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Feb. 2017</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>May 2017</td>
<td>0</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Aug. 2017</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

2.1.4 DNA extraction

The QIAGEN DNeasy Blood & Tissue Kit and the QIAGEN DNeasy Plant Mini Kit (QIAGEN, Tokyo, Japan) were both used to extract DNA from the tissue samples. The only reason the QIAGEN DNeasy Blood & Tissue Kit was used at all was because it had previously been used to extract DNA from giant clams (Pappas et al., 2017). However, when data from Sanger sequencing using the DNA extracted with these kits proved to be lacking in resolution, and the use of NGS became the main priority, all DNA was then extracted using the QIAGEN Mini Plant Kit, which provided higher quality DNA for use in NGS. The protocol as described in each kit was followed and extracted DNA from both kits was then stored in separate 96-well plates at -20°C.

The DNA extracted using the QIAGEN DNeasy Blood & Tissue Kit came from samples collected in only three of the four collection periods, November 2016, February 2017, and May 2017. DNA from all tissue samples collected for the seasonal tridacnid sampling (107 total from four sampling periods) was extracted following the QIAGEN Plant Mini Kit protocol. For the QIAGEN Plant Mini Kit, frozen tissue samples stored at -80°C were ground into a fine powder using liquid nitrogen and a mortar and pestle for the lysing of the tissue. Modifications to the QIAGEN Plant Mini Kit protocol were made to improve the DNA concentration. Those
changes were as follows: the addition of 0.5 ml of glass beads to the 1.5 ml centrifuge tube with the buffer AP1 and RNase A (volumes of which were not changed from original protocol) to aid in the breakup of the cells, and the increase of the duration of the incubation step from 10 min to 20 min, also to aid in the lysis of the cells. The final DNA was then stored in 96-well plates at -20° C.

2.1.5 Sequencing

Both Sanger sequencing and Next Generation Sequencing were used to sequence target gene regions of the *Symbiodinium* DNA. Only Sanger sequencing was used to sequence the 16s mitochondrial gene locus of the tridacnid samples to ensure correct species identification. DNA that was sequenced with Sanger sequencing was extracted with the QIAGEN Blood & Tissue Kit. The primer set 16Sar (F) (5′-CGC CTG TTT ATC AAA AAC AT-3’) and 16Sbr (R) (5′-CCG GTC TGA ACT CAG ATC ACG T-3’) (Sue et al., 2014) was used to isolate this region following the same protocol as my previous study (Pappas et al., 2017). The polymerase chain reaction (PCR) protocol for isolating the 16S gene locus was as follows: initial 95 °C for 15 min to activate the QIAGEN mix, 30 cycles of 94 °C for 6 min to denature the DNA, 53 °C for 1 min to anneal the DNA, 72 °C for 1 min for elongation of the DNA, and a final elongation step at 72 °C for 10 min. Sanger sequencing was also used to sequence the ITS-rDNA gene region of the *Symbiodinium* DNA using the primer pair zITS (F) (5′-CCG GTG AAT TAT TCG GAC TGA CGC AGT-3’) and ITS4 (R) (5′-TCC TCC GCT TAT TGA TAT GC-3’) (Reimer et al. 2006), also following the same previous study (Pappas et al., 2017). The PCR protocol for the ITS region was as follows: 95 °C for
15 min (activation), 35 cycles of 94 °C for 30 sec (denaturation), 51 °C for 45 sec (annealing), 72 °C for 2 min (elongation), and 72 °C for 10 min (final elongation). After PCR, samples were run in 1% agarose gel under 90 V for 45 min and examined under UV light. Samples were then cleaned by incubating with exonuclease I and FastAP™ Thermosensitive Alkaline Phosphatase (ExoFAP; USB, Cleveland, OH, USA) at 37°C for 60 min, followed by 85°C for 15 min, and then sent for Sanger sequencing at the KAUST Bioscience Core Lab (BCL). Sanger sequencing is known to be adequate when determining the species of the clam and the general clade and type of *Symbiodinium*, but is not reliable when trying to detect potential shifts in more specific *Symbiodinium* types. For that reason, Next Generation Sequencing and the SymPortal database analysis was used to determine all present *Symbiodinium* types within a sample and if a shift in *Symbiodinium* types occurred across seasons.

Next Generation Sequencing or metabarcoding was performed on an Illumina Miseq platform. The ITS2 gene was isolated using the primer set ITSintfor2 (F) (5’-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3’) with overhang adapter sequences underlined (LaJeunesse, 2002) and ITS2-reverse (R) (5’-GTCTCGTGGGCTCGGAGATGTGTATAAGACAGAGGATCCATATGCTTAAGTTTCAGCTG GGT-3’) with overhang adapters underlined (LaJeunesse, 2002), which has been used to isolate the ITS2 gene region in *Symbiodinium* from corals and anemones (LaJeunesse and Trench, 2001; LaJeunesse, 2002). The QIAGEN Multiplex PCR Kit (QIAGEN, Tokyo, Japan) was used for PCR. For PCR preparation, each sample well contained 2 µl of extracted DNA, 2 µl of each primer, 11 µl of QIAGEN Mastermix,
and 9 µl of DI water for a total volume of 26 µl in each well. The PCR protocol for isolating and amplifying the ITS2 gene was as follows: 95° C for 15 min to activate, then 40 cycles of 90° C for 30 sec, 51° C for 30 sec, and 72° C for 30 sec, ending with 72° C for 10 min. The PCR product was then checked with the automated gel electrophoresis Qiaxcel machine for all samples. After repeating the PCR process twice more and obtaining ~ 78 µl of PCR product per sample, PCR product was pooled into two 96-well plates and DNA was purified with Agencourt AMPure XP magnetic bead system (Beckman Coulter, Brea, CA, USA). Nextera XT indexing and sequencing adapters were added via PCR with the following protocol: 95° C for 15 min for activation, then eight cycles of 96° C for 30 sec, 56° C for 30 sec, and 72° C for 30 sec, ending with 72° C for 10 min (8 cycles, total PCR cycles for all samples = 48) following the manufacturer’s instructions. The resulting DNA samples were normalized to the same concentration using the SequalPrep Normalization Plate (96) Kit. A final volume of 20 µl of eluted DNA per sample was then used for the libraries. Two libraries were created from the two plates of samples (151 samples total). Each library had a total volume of ~2 ml, which was then concentrated to ~50 µl for loading into the Miseq platform. The quality of the libraries was checked and quantified with the Agilent 2100 Bioanalyzer and Agilent QPCR NGS Library Quantification kit (Agilent Technologies, Santa Clara, USA) before loading into the Miseq platform. The pooled libraries were sequenced at 8pM with 10% phiX on the Illumina MiSeq, 2 × 300 bp paired-end version 3 chemistry according to the manufacturer’s specifications. Data was sent to the BCL. Final fasta files were created and then analyzed in the SymPortal system (Hume et al., unpublished) to
determine the proportion of *Symbiodinium* types in each sample. SymPortal is a database and data analysis program designed to identify all divergent ITS2 sequences in a given set of sequences. SymPortal is able to run a large amount of NGS-derived sequence data and determines how many divergent intragenomic variants (DIVs) are present within the given set of sequences. From there, it is able to identify the number and abundance of unique type profiles. Type profiles are unique combinations of DIVS that occur in the current dataset often enough to distinguish them as significant, meaning that they are most likely not results of mutation or sequencing mistakes that occur due to the multi-copy nature of the ITS2 gene. Data analyzed with the SymPortal and information on environmental and ecological variables can help determine if these type profiles are biologically different from each other.

### 2.1.6 Data analysis

For samples sequenced by Sanger sequencing, sequences were analyzed, trimmed (445 bp for 16S and 658 bp for ITS), and edited using the program Geneious R8 (Biomatters Ltd., Auckland, New Zealand). A BLAST search on GenBank was performed to confirm species identity for tridacnid sequences. The Median-joining haplotype network showing the relationships amongst the haplotypes was generated in NETWORK v4.6.1.3 (Bandelt et al. 1999). Final editing of the haploweb was done with Adobe Illustrator (Adobe Illustrator CC version 2015.3). After the haploweb was created, all unique haplotypes were examined for patterns based on month and reef type. Divergences between each haplotype occurred in the ITS2 region of the entire ITS gene region, which included the ITS1, 5.8s, and ITS2 gene
regions. Because divergences were seen in the ITS2 region, NGS sequences data based on the ITS2 region alone was used to examine the differences in more detail.

Sequences from NGS were analyzed by the SymPortal database and analyzer developed by Benjamin Hume in Christian Voolstra's lab at KAUST. The system was able to determine the proportion of each *Symbiodinium* type profile in each sample. Visual representation of the data was then created with the data from Next Generation Sequencing using Microsoft Excel with final edits done in Adobe illustrator (Adobe Illustrator CC version 2015.3).
2.2: Experimental Bleaching Methods

2.2.1 Experimental design

To determine the stability of the tridacnid holobiont during and after bleaching stress, 14 individual *T. maxima* clams were collected and placed under experimental thermal stress in the laboratory to induce bleaching. Analyzed samples were collected at three time periods: pre-bleached, bleached, and post-bleached (see Fig. 2b).

2.2.2 System setup

Seven tanks in the CMRCL SeaLab facility at KAUST were filled with raw seawater at an ambient temperature of 27°C in March 2017. The tanks were connected to a flow-through system with intake seawater sourced about one kilometer away from the coast. Due to the flow-through of ambient seawater, control tanks were not able to truly represent a control temperature as the ambient temperature was not controlled and naturally increased throughout the duration of the experiment (March – August 2017). The flow-through system ran for four days before clams were placed inside. Each tank held two clams with one tank designated as the control. In total, there were six replicates designated for thermal treatment. HOBO temperature loggers were placed in each tank to record the temperature every ten minutes for the duration of the experiment.

2.2.3 Collection of individuals

Fourteen *T. maxima* clams were collected at Abu Shosha, an inshore reef part of the seasonal sampling project on March, 28, 2017. The water temperature at
collection depth was 27°C. All clams were identified in the field as *T. maxima* and were of similar length (14-22cm) and from similar depth (5-10m). All individuals were first sampled in the field in the same way as previously described in the seasonal tridacnid sampling project (see section 2.1.3). After tissue collection, each clam was removed from the reef by either simply lifting it from the substrate or by carefully lifting and cutting the byssal threads with a knife. The clam was then temporarily numbered using labeled tags until transferred to the SeaLab facility. Samples from the field were rinsed with Milli-Q water, cut in half, and stored in 96% ethanol (half of the sample) or flash-frozen with liquid nitrogen (half of the sample) for storage at -80°C. Once placed in the experimental tanks, clams retained their identification numbers to allow for a continuous sampling and observation of symbiont community shift by individual.

2.2.4 Acclimation

All individuals were held in ambient raw seawater at 27°C inside the tank setup for approximately two weeks before beginning temperature manipulations (following Leggat et al., 2003)(see Fig. 2b). Clams were sampled two days before treatments began, directly after the acclimation period, to represent an acclimated, healthy, and pre-bleached sample. Sampling in the SeaLab followed the same sampling protocol used in the field as described in the seasonal tridacnid sampling (see section 2.1.3). Photos were taken at this time and throughout the experiment to record visual changes.
2.2.5 Thermal treatments

Mortality of two individuals (13 & 14) occurred during the acclimation period, changing the treatment number to five tanks with two replicates per tank and one tank with two replicates for the control. The control tank would receive ambient seawater that increased gradually and naturally from 27°C in March to 32°C in July and August. At this point, five tanks were designated as treatment tanks, two of which would receive a temperature increase of 2°C and the other three tanks would receive an increase of 4°C. Water was heated using aquarium heaters to reach desired temperatures. However, the aquarium heaters available (60 watts) were not able to reach the desired temperature for the plus 4°C treatment. The design was then changed to all treatment tanks (5) under a plus 2°C treatment. With the naturally increasing ambient seawater temperature, the treatment temperature was checked every other day to maintain 2°C above the ambient seawater temperature. By the end of July, the treatment temperature was set to 34°C. Ambient temperature reached 32°C during July and August, the hottest period during the experiment. After two weeks, with all treatment tanks set at 34°C, and control tanks at 32°C, heaters were removed to encourage symbiont recovery. Two weeks after removing the heaters, the temperature in all tanks was approximately 32°C. At this point, to aid in bleaching recovery, filtered seawater at 27°C was slowly pumped into all tanks including the controls. After two weeks of the continuous addition of 27°C seawater, the experiment was determined to be finished. HOBO temperature loggers recorded temperature every 10 min for the entire duration of the experiment. See Figure 2 for a visual summary of the bleaching timeline.
2.2.6 Sampling frequency

A previous study on giant clam bleaching in aquaria (Leggat et al., 2003) collected tissue samples from each individual every week during an eight-week experiment. However, because my experiment duration was longer than eight weeks and I suspected sampling stress could potentially cause mortality, sampling frequency in my experiment was limited to three periods, one directly after the acclimation period (April 13\textsuperscript{th}), one during July when temperatures and bleaching severity were at their highest (July 11\textsuperscript{th}), and one in August (August 13\textsuperscript{th}), directly before ending the experiment (see Fig. 2b). These three periods were to represent healthy or pre-bleached, bleached, and post-bleached or recovered clam holobionts. Although these three sampling periods were chosen to represent an accurate bleaching status of the sampled individuals, it is important to note that some clams sampled during the “bleached” period did not show signs of bleaching and some clams sampled during the “post-bleached” period had not yet recovered from bleaching. Also, it is important to note that all bleaching statuses were determined visually and qualitatively with a defined set of criteria (Fig. 3). Photos were taken every week to record visual changes and comparatively assess bleaching progress.

2.2.7 Tank maintenance

Using raw seawater meant that fouling and interference of other organisms would occur. For this reason, cleaning of tanks was performed every second to third day during the experiment. Cleaning involved siphoning debris from the tank and scrubbing the walls to rid the tank of excess algae. Animal stress was taken into consideration when cleaning the tanks, and cleaning was done to create as little
disturbance to the clams as possible. The overall health and stress level of the clams was a higher priority than a clean tank. Therefore, on sampling days and days of high stress due to increased temperatures or flow-through shut offs, cleaning was not done to decrease the amount of stress on the clams.

2.2.8 Bleaching and recovery

Once visible signs of bleaching were present (July 1st), which meant any detectable loss of color, either manifesting as overall paling or white patches (see Fig. 3), photos were taken every two to three days to record a rough progression of bleaching. Photos in Figure 3 show healthy clams (photos taken the second week of April), slightly bleached clams (photos taken the first and second week of July), moderately bleached clams (photos taken July week 2), and severely bleached clams (photos taken July week 2 and 3). Samples representing the bleached holobiont were taken from clams on July 11th. On July 23rd, the temperature was gradually decreased to the ambient temperature by removing the heaters. All tanks remained at approximately 32°C for ten days with no manipulation of temperature. Due to the lack in visible signs of recovery during these ten days, filtered seawater at 27°C was gradually and continuously pumped into all tanks for two weeks. After these two weeks, most clams showed signs of recovery, and all were sampled at this point (August 13th) to represent the recovered tridacnid holobiont. Four days after clams were sampled (August 17th), all were returned to the sheltered side of the collection reef between 5-10 m and specifically placed between coral colonies to keep them upright and sheltered from wave energy.
2.2.9 DNA extraction

All sample analysis and lab work (seasonal tridacnid sampling and experimental bleaching sampling) to be processed for NGS were started at the same time (August 18th), and the protocol for extracting the DNA from the *Symbiodinium* as well as for isolating the gene region ITS2, follows the same protocol as listed in the seasonal tridacnid sampling (see section 2.1.4). For the experimental bleaching project, DNA was extracted from all samples, including the samples from the field, samples of pre-bleached clams (taken April 13th), samples of bleached clams (taken July 11th), and samples of recovered clams (taken August 13th). DNA was extracted following the QIAGEN Plant Mini Kit protocol with modifications as described in the seasonal tridacnid sampling methods above (see section 2.1.4). Extracted DNA was stored in 96-well plates at -20°C. The QIAGEN DNeasy Blood & Tissue Kit was not used for any of the samples from the experimental bleaching project since all DNA was already extracted with the QIAGEN Plant Mini Kit, making the use of the QIAGEN DNeasy Blood & Tissue Kit redundant.
Figure 2. Results from an aquaria-based bleaching experiment. (A) Timeline with photos of different individuals during the experiment. (B) Timeline showing horizontal bars representing each individual in the experiment with colors representing bleaching appearance and lines representing important dates during the experiment. (C) Temperature profiles for the ambient seawater temperature, the average treatment temperature, and the average temperature for Abu Shosha (the collection site) during this period taken from 2015 (NOAA SST data).
Table 3. Photos taken during the bleaching experiment and the criteria for defining bleaching status indicated qualitatively and visually in this study. Status was defined as (A) healthy, (B) slightly bleached, (C) moderately bleached, or (D) severely bleached.

<table>
<thead>
<tr>
<th>Status</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Healthy: No signs of visible bleaching</td>
</tr>
<tr>
<td>B</td>
<td>Slightly bleached: Early stage of bleaching where the first signs of bleaching can be observed as a general paling of the mantle tissue color starting either from the center of the clam or the edge of the mantle tissue</td>
</tr>
<tr>
<td>C</td>
<td>Moderately bleached: Bleaching that has increased in intensity and/or area covered over healthy mantle tissue manifesting as either overall paling or pronounced white patches.</td>
</tr>
<tr>
<td>D</td>
<td>Severely bleached: Last stage of bleaching showing distinct white patches covering 50% or greater of the surface area of the mantle tissue</td>
</tr>
</tbody>
</table>

Figure 3. Photos taken during the bleaching experiment and the criteria for defining bleaching status indicated qualitatively and visually in this study. Status was defined as (A) healthy, (B) slightly bleached, (C) moderately bleached, or (D) severely bleached.
2.2.10 PCR protocol

Only *Symbiodinium* DNA was prepared for analysis from the samples of the experimental bleaching project. Tridacnid DNA was not necessary to extract due to positive identification in the field of tridacnid species. The ITS2 gene was isolated and amplified using the same primer set ITS2F Miseq (F) and ITS2rev (R) following the same methods as the seasonal tridacnid sampling component (section 2.1.5).

2.2.11 Sequencing & data analysis

Only NGS was used for the bleaching experiment samples due to its high resolution in detecting *Symbiodinium* types and changes. NGS was performed by the Miseq system following the same protocol as described in the seasonal tridacnid component (section 2.1.6). The SymPortal database was then used to analyze the data from the bleaching experiment samples. Microsoft Excel and Adobe Illustrator were used to create the graphs.

2.2.12 Bleaching temperature data analysis

As defined by NOAA, the bleaching threshold (BT) of corals is 1°C above the maximum monthly mean determined from SST data over a period of >50 years (Eakin et al., 2009). HotSpots are defined as the periods when the SST is above the MMM in a region. Corals experience accumulated thermal stress that is not necessarily represented by the current SST or HotSpot temperatures. Thus, degree heating weeks, calculated by summing the previous 12 weeks of SSTs above the bleaching threshold for a given point in time, indicate the accumulated thermal stress. For example, NOAA has determined that significant bleaching in corals
happens at 4°C-heating weeks. This means that the corals in the given region and at a certain time point have experienced thermal stress equivalent to 4°C above the maximum monthly mean in the previous 12 weeks. DHW is a summation of the SSTs above the BT in 24 half-week time steps. When a new half week SST is measured, the earliest half week SST is dropped from the running sum, determining the DHW for a given time. The MMM in this project was determined from NOAA SST data for Abu Shosha specifically. The bleaching threshold for clams was calculated by conserving the 4°C-heating week measurement at which significant bleaching is observed in corals. The point at which significant bleaching was observed in the experimental bleaching project for tridacnids was July 9th. Determining that the summation of SSTs above the BT for the 12 weeks prior to July 9th was 4°C, the BT was then able to be calculated.

Conserving the measurement that represents the accumulated thermal stress (DHW) and solving for the bleaching threshold is the most logical in terms of comparing corals and clams and their tolerance to increasing temperatures. However, because data available to the scientific community, policy makers, and the public is the universal dataset of SST from NOAA and DHW maps as determined by the bleaching threshold for corals, the bleaching threshold for corals and the DHW based on this threshold was calculated as well for the clams in the bleaching experiment. By conserving the bleaching threshold at 31°C for Abu Shosha and solving for the DHW, would allow for an easy comparison between this data and the DHW provided by NOAA to be able to predict giant clam bleaching for Abu Shosha based on the thermal tolerance scale of corals. Given that coral bleaching is a larger
threat than clam bleaching and has stronger implications for the ecosystem, it is best that NOAA calculated DHW based on the thermal tolerance of corals, however, it is important to note, that the tools provided by NOAA are optimized to predict bleaching in the coral holobiont and may not be suited to best predict bleaching in other photosymbiotic holobionts.
3. RESULTS

3.1 Seasonal Tridacnid Sampling Results

Sanger sequencing results showed that all (30) sampled tridacnids of the seasonal tridacnid sampling were *Tridacna maxima* with a 99% match to either the GenBank sequence derived from 16s mitochondrial DNA of *T. maxima* from the Red Sea (AM909742) (Richter et al., 2008) or the coral triangle (EU341335) (DeBoer et al., 2008). Sanger sequencing data for *Symbiodinium* sequences was only able to show the dominant *Symbiodinium* type, with all samples matching to the species *S. microadriaticum* a.k.a. *S. sp. CCMP828* (LK934672) a.k.a. A1 in Clade A (Lee et al., 2015).

A median-joining haplotype was created using the sequences derived from Sanger sequencing data of the samples from November 2016, February 2017, and May 2017. The ITS haplotype (Fig. 4) did not indicate any pattern based on collection month or reef type. However, the haplotype (Fig. 4) was able to show that multiple haplotypes exist in the *Symbiodinium* samples. Haplotype differences derived from variation in the ITS2 sequence of the ITS region, which was checked by examining the sequence variations and their location within the ITS region (ITS1, 5.8s, and ITS2) on Geneious. The variation found in the ITS2 region suggests a more detailed examination of these differences could be observed by the execution of NGS data and analysis in the SymPortal using only the ITS2 region. Because Sanger sequencing results were examined after processing samples from May 2017, and were found to lack resolution in examining the ITS2 region, NGS was then chosen to represent all samples in the project.
Figure 4. Haplotype network of ITS sequences from Sanger sequencing of *Symbiodinium* samples from November 2016, February 2017, and May 2017. Colors represent (A) month and (B) reef type.
Further analysis of *Symbiodinium* types using NGS revealed many specific types within Clades A, C, and D in the sample set and was able to detect *Symbiodinium* types at levels as low as 0.1%. SymPortal analysis found 99 DIVs or divergent intragenomic variants in the ITS2 sequences. Of these DIVs, the majority belonged to Clade C (58) followed by types in Clade D (26) and then Clade A (6). Of those 99 DIVs detected, 57 type profiles were found. As described in the methods section (see section 2.1.5), type profiles are unique combinations of DIVs that occur frequently enough in the dataset to be distinguished as legitimate combinations rather than mutations representing one type due to the multi-copy nature of the ITS2 gene. Of the 57 unique type profiles, five were combinations of A1 types, each of which dominated at least one sample in the sample set. Only type A1 type profiles were found to be dominating type profiles in the sample set. Type profiles belonging to Clades C and D, although more diverse than Clade A type profiles, were only found as background type profiles with proportions no greater than 26%. There were three unique type profiles belonging to type A1 that were present as the dominant types in the majority of samples (Fig. 5-7). Type profiles A1-A1aw and A1-A1av are the most common dominant type profiles present in the seasonal tridacnid samples (Fig. 5, 6), with the pure A1 type present as a common dominant type in the bleaching experiment samples (Fig. 7), results of which are presented in the following section.

With regards to the background or rarer type profiles present in the samples, types of Clade C and Clade D were found, but only ever as background
types (see Appendix: Fig. 1), which supports the Sanger sequencing data showing the dominant type as A1 for all samples. The background types belonging to Clade C and D and their proportions differ across collection month, reef type, and individual, but no pattern is present for those rarer types. Both month and reef type had a significant effect on the overall abundance of types per sample (Table 2), but the main pattern that can be seen in Figures 5 and 6 is how the two A1 type combinations, A1-A1aw and A1-A1av, differ as the dominant type profile based on reef type (Fig. 6). The inshore reef, Abu Shosha had the majority of samples dominated by the A1-A1aw type profile, while the mid shelf and offshore reefs (Shark Reef and Shib Nazar) were mainly dominated by the A1-A1av type profile (Fig. 6). Figures 5 and 6 show the proportion of type profiles within each sample shown by colors, however, the key in these figures only shows the colors of the most dominant type profiles present. For a more comprehensive key for this dataset, see Appendix, Figures 2 – 4. Statistical and environmental data help to determine if these different type profiles are significant.

A perMANOVA test (Table 2) using the Bray Curtis distance method and 999 permutations in the R vegan package (Oksanen, 2011) was done. Reef type and month had a significant effect on the overall abundance of *Symbiodinium* types in each sample, but the interaction between the two was not significant in determining overall abundance. However, table 3 shows percentages of the three most common dominant type profiles within the entire dataset, showing the high correlation of the inshore reef type to *Symbiodinium* type profile A1-A1aw and the bleached status to *Symbiodinium* type profile of A1.
The temperature profiles for the three sampling reefs (SST data from NOAA) were similar to the profiles for temperature in previous years (Roik et al., 2016), suggesting that the shifting of *Symbiodinium* type profiles in tridacnids shown in these results represent a baseline of normal annual shifting. There is no pattern seen in the data organized by season, suggesting that seasonal stability is more likely than seasonal shifting (Fig. 5). Individuals harboring type profiles A1-A1aw tend to remain stable with this relationship even in different time periods of the year and this is true for those harboring A1-A1av. Reef type has a greater effect on *Symbiodinium* type profile dominance than season as shown by the clear structure of the data when organized by reef type (Fig. 6).

Mortality of some of the marked individuals occurred at all three reefs over the year of sampling. Shib Nazar lost one out of the original 10 before the August 2017 sampling date. Shark Reef lost six out of the 10 before the May 2017 sampling date. Abu Shosha lost four of the 10 before the May 2017 sampling date (see Table 1). When mortality occurred, a collection of tissue samples from nearby clams were taken for representation of the holobiont for the given month to maintain a similar sample size. The clams sampled that were not originally tagged in November showed similar type profile dominance as the others of the same reef type. This indicates that reef type has a greater effect on *Symbiodinium* type profile over season and individual.
Figure 5. Proportion of *Symbiodinium* types of samples from the seasonal tridacnid sampling organized by month/season. The key shows colors for only the most dominant types, type A1-A1av, A1-A1aw, and A1.
Figure 6. Proportion of *Symbiodinium* types of samples from the seasonal tridacnid sampling organized by reef type. The key shows colors for only the most dominant types, type A1-A1av, A1-A1aw, and A1.
<table>
<thead>
<tr>
<th></th>
<th>Df</th>
<th>SumsOfSqs</th>
<th>MeanSqs</th>
<th>F. Model</th>
<th>R²</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Month</td>
<td>4</td>
<td>2.4258</td>
<td>0.60646</td>
<td>3.8690</td>
<td>0.12578</td>
<td>0.001***</td>
</tr>
<tr>
<td>Reef Type</td>
<td>2</td>
<td>2.2154</td>
<td>1.10770</td>
<td>7.0668</td>
<td>0.11487</td>
<td>0.001***</td>
</tr>
<tr>
<td>Month:</td>
<td>6</td>
<td>0.6942</td>
<td>0.11570</td>
<td>0.7382</td>
<td>0.03600</td>
<td>0.790</td>
</tr>
<tr>
<td>Reef Type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residuals</td>
<td>89</td>
<td>13.9506</td>
<td>0.15675</td>
<td></td>
<td>0.72335</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>101</td>
<td>19.2860</td>
<td></td>
<td></td>
<td>1.00000</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. PermANOVA statistical test values for determining effect of month and reef type on total abundance of *Symbiodinium* types within samples. The last column shows significance if the P value is less than 0.001.
3.2: Experimental Bleaching Results

3.2.1 Bleaching timeline, temperature profiles, and mortalities

The 2°C plus treatment temperature did induce bleaching, but only after the ambient seawater temperature reached 32°C. For a detailed visual representation of thermal regime, mortality, bleaching status, and sampling dates, see Figure 2.

Unfortunately, out of the 10 individuals in treatment tanks, three of them died before the end of the experiment (see Fig. 2b). The first two mortalities occurred during the acclimation period and the last three mortalities occurred after visible signs of bleaching were present during the thermal regime. As shown by the thermal treatments, tridacnid clams bleached at approximately 34°C. Recovery for the clams that did survive took approximately two weeks after ending the thermal treatments, but only after drastically decreasing the temperature to 27°C. After bleaching was observed and a sample was taken from each individual, the recovery process started. Heaters were removed and the temperature in treatment tanks decreased to 31 - 32°C, which was the temperature of the ambient seawater at the time (July). After two weeks of ambient sea water temperatures of 32°C, the control clams began to show signs of bleaching. At this point, filtered seawater at 27°C was pumped into all tanks. Only when the temperature dropped to 27°C were the clams able to recover. It is important to note that this quick decrease in temperature does not reflect what naturally occurs in the field. The bleaching of the controls was able to show that all clams in the experiment bleached at a similar thermal intensity. The control clams bleached when exposed to 32°C for four weeks and the treatment clams bleached when exposed to 34°C for two weeks. However, to avoid further
bleaching in the control clams, temperature was decreased by the use of filtered and chilled seawater. Figure 2b shows temperature for the treatment tanks at approximately 27°C from August 2nd until the end of the experiment. The decrease in temperature resulted in the recovery of three bleached clams and partial recovery in the other four of the treatment clams.

Temperature profiles for all tanks in the experimental setup were created and analyzed. The temperature profile for the control tank shows a steady increase from 27°C in March to 31°C in August (Fig. 2c). Here it can be seen that between July and August, when temperatures were at the maximum, high temperatures lasted until the end of the experiment, which resulted in the slight bleaching of the control clams. Figure 2 also shows the temperature profile of the treatment tanks as an average of the five tanks. The photos in Figure 2a depict the visual change over time of the tridacnids at various time periods (they serve as visual representation only as they do not come from the same individual). Photos show healthy clams *in situ* at the beginning of the experiment and a recovered clam at the end.

3.2.2 Calculation of the bleaching threshold and DHW

Following the calculations and standards of the MMM, bleaching threshold, and DHW of NOAA. Figure 2 shows that clams started to bleach on July 1st. To calculate the bleaching threshold of Red Sea tridacnids, the DHW at which significant bleaching is observed in corals was held as a constant and the bleaching threshold was solved for. The point at which significant bleaching was observed in clams in this experiment was July 9th. Thus, the accumulated stress for the previous 12 weeks before July 9th was determined to be 4°C-heating weeks. For this to be
true, the bleaching threshold had to have been 32°C for tridacnids in this experiment. Figure 7 shows the temperature profiles for all measurements involved in the calculations of DHW.

Because DHW is the measurement that best predicts bleaching in coral reefs, it was held as the constant in these calculations. If 4°C-heating weeks is determined to be the threshold at which we can expect significant bleaching in corals, it should be the same for clams. However, if we keep DHW constant, the bleaching threshold will change, and that was what was calculated here. However, because SST and DHW data from NOAA are what is readily available to the public, it is important to calculate DHW for clam bleaching based on the bleaching threshold of corals. This calculation of DHW while keeping BT constant will allow for easier comparison when looking at NOAA coral bleaching prediction datasets. The DHW calculated as if the bleaching threshold was 31°C shows that clams started to bleach at 15°C-heating weeks and severe bleaching in clams was seen at 22°C-heating weeks. It is important to note that calculations for bleaching thresholds and DHW are optimized for the coral holobiont, and therefore may not as accurately predict bleaching in clams, but these calculations may serve as rough prediction measurements for tridacnid bleaching in the Red Sea.
Figure 7. Temperature profiles representing the average treatment temperature, the monthly mean of the ambient seawater temperature, the maximum monthly mean, and the bleaching threshold according to the temperatures recorded during the bleaching experiment.
3.2.3 Changes in the holobiont *Symbiodinium* proportions

Next Generation Sequencing shows that the *Symbiodinium* type profiles did shift due to thermal stress. Samples of bleached clams show an overwhelming dominance of type A1 *Symbiodinium* (Fig. 8). Healthy, pre-bleached clams have type A1-A1aw as the dominant type (Table 3), which is the same type found in the highest abundance in the majority of clams from the seasonal tridacnid samples of the inshore reef. The clams collected for the experimental bleaching were from Abu Shosha, the same inshore reef with samples most commonly dominated by the A1-A1aw type profile.

The type profile shift from either A1-A1aw or A1-A1av to A1 only occurred after the clam started to show signs of bleaching. Of the clams that recovered completely (3), all returned back to either a dominance in A1-A1aw or A1-A1av. Clams that partially recovered (4), did not show a shift in dominance from A1 to the other type profiles. Although type A1 is the most common dominate type in bleached samples, it was present as a dominant type in field samples, of which none showed visible signs of bleaching. Furthermore, all dominant A1 type profiles (A1-A1aw, A1-A1av, and A1) were found in every reef type, every season, and every sampling period during the bleaching experiment.
<table>
<thead>
<tr>
<th>Dominant type profile</th>
<th>Reef type</th>
<th>Inshore</th>
<th>Mid shelf</th>
<th>Offshore</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1-A1aw</td>
<td></td>
<td>77%</td>
<td>20%</td>
<td>12.5%</td>
</tr>
<tr>
<td>A1-A1av</td>
<td></td>
<td>10.4%</td>
<td>57%</td>
<td>60%</td>
</tr>
<tr>
<td>A1</td>
<td></td>
<td>6.25%</td>
<td>11.42%</td>
<td>7.5%</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td>6.35%</td>
<td>11.6%</td>
<td>20%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dominant type profile</th>
<th>Bleaching status</th>
<th>Healthy</th>
<th>Bleached</th>
<th>Post-bleached</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1-A1aw</td>
<td></td>
<td>75%</td>
<td>14%</td>
<td>50%</td>
</tr>
<tr>
<td>A1-A1av</td>
<td></td>
<td>16.6%</td>
<td>7%</td>
<td>25%</td>
</tr>
<tr>
<td>A1</td>
<td></td>
<td>4.2%</td>
<td>79%</td>
<td>25%</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td>4.2%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Table 3. Percentage of dominant type profiles per samples by reef type and by bleaching status.
Figure 8. Proportion of Symbiodinium types of samples from the bleaching experiment organized by bleaching status. The key shows colors for only the most dominant types, Type A1-A1av, A1-A1aw, and A1.
4. DISCUSSION

Stability in the holobiont system is advantageous and allows the organisms to evolve together in response to changes in the environment. A species-specific symbiotic relationship is advantageous in that it may allow for a high acclimatization potential or local adaptation of a given type of *Symbiodinium* within that host (Goulet, 2006) and many corals show stability rather than symbiont shuffling (Goulet & Coffroth, 2003; Thornhill et al., 2006; Stat et al., 2009; Pettay et al., 2011). However, when the environment changes rapidly, like it does during a bleaching event, the holobiont needs to be able to respond quickly enough to ensure its survival. In the cases of drastic changes over short periods of time, it may be more advantageous for the holobiont to be able to shift its symbionts. This has been shown to happen in corals (Rowan and Knowlton 1995; Rowan et al. 1997; Glynn et al. 2001; LaJeunesse et al. 2004; van Oppen et al. 2005; Stat and Gates 2010), but other photosymbiotic marine invertebrates have not been so extensively studied. The first record of symbiont shuffling in the Red Sea giant clam holobiont in this study shows that shifting does occur in clams as well, and may have implications in bleaching resistance or tolerance.

Previous studies of *Symbiodinium* diversity and shuffling in holobionts have recently been able to identify specific types within clades on more detailed levels (Rodriguez-Lanetty, 2003; Correa and Baker, 2008; Stat et al., 2011; Quigley et al., 2014), but have only been able to do reach the highest level of detail with microsatellites (Santos et al., 2004; Finney et al., 2010; Pettay et al., 2011) that need to be designed for specific clades. The issue of the ITS2 as a marker in determining
different types comes from its multi copy nature, which may overestimate the number of different types within a sample. Fortunately, SymPortal is able to determine the likelihood that a variant is due to a multi copy mutation and thus, identifies only those variants that are most likely true variants. This program will allow for faster progress in determining *Symbiodinium* diversity, which then will help in identifying functions of different types.

As shown in this study, Red Sea giant clams have a fairly fixed relationship with the dominant clade (Clade A) and type (A1) of the *Symbiodinium* they host, supporting earlier work on clams in this region (Pappas et al., 2017). Clade A type A1 is fairly common in corals of the central Red Sea as well (Sawall et al., 2014; Ziegler et al., 2017) and has been reported to be a common clade harbored by giant clams in other regions (Belda-Baillie et al., 1999; Baillie et al., 2000a, 2000b; DeBoer et al., 2012; Ishikura et al., 2017). The diversity amongst clades present in one clam individual is not of the same magnitude as found in corals, which can be found to harbor up to three different clades within one colony (see Baker 2003). Results from my data found that both Clades C and D exist in many samples, but only as background types no greater than 26%. The lack of cladal diversity as dominant types in Red Sea tridacnids may be due to the fact that giant clams are not as spatially diverse in relation to corals, therefore needing a less diverse set of *Symbiodinium*. In the project sampling region, only two species of tridacnids were found (Pappas et al., 2017), both of which have been found no deeper than 20 - 25m (see Junio et al., 1989; Gilbert et al., 2007; Pappas et al., 2017), whereas hermatypic corals can be found as deep as 100m in the Red Sea (Fricke & Schuhmacher, 1983).
The depth profile of giant clams has been shown to influence the diversity of symbiotic algae, with *T. squamosa*, the more spatially diverse of the two species, harboring both Clades A and C, while *T. maxima*, found on shallow reefs (<10m depth) only harboring Clade A (Richter et al., 2008). A study done in corals showed that depth and geographic location rather than host species played a larger role in symbiont community (Finney et al., 2010). This is similar to my results showing specific communities based on reef type rather than season. Another difference between corals and clams that may explain why we do not see a large diversity amongst clades in clams is that corals are colonies made up of many individual polyps and usually form microhabitats that can be niches for different types of *Symbiodinium* (Rowan et al., 1997; Stat et al., 2011). Giant clams, however, are only one animal and do not form as many different niches for specialized *Symbiodinium* types.

The relationship with Clade A in the Red Sea tridacnids may be due to its association with shallow-dwelling hosts, most likely due to its heat and stress tolerance (reviewed in Baillie et al., 2000b). All samples in this project came from *T. maxima* giant clams, therefore the lack of other dominant clades in the symbiont community is not surprising and can potentially be explained by their specific depth range and irradiance levels. However, harboring only Clade A as the dominant clade does not mean that the holobiont lacks *Symbiodinium* diversity. The results from this study show that even within the type A1, aka, *Symbiodinium microadriaticum*, there are three separate type profiles that are distinctly separated by reef type and bleaching status in giant clams. These results are similar to results from Baillie et al.
where multiple divergent types within A1 were present within *Symbiodinium* isolates from giant clams in the Indo-Pacific. They were able to determine that divergent types within type A1 were found across multiple species of giant clams, individuals within the same species, and within one individual host (Baillie et al., 200b). However, their examination of these differences by use of RAPD fingerprints was not able to identify the types to which these divergences belong (Baillie et al., 200b). Results of my study support the earlier work on type A1 diversity seen within giant clams and show more detail in the pattern and type of these divergences. Baillie et al. (2000a), using the ITS gene region as a marker, suggest that giant clams host multiple distinct types within type A1. They were also able to determine that the A1 types derived from giant clams were different than the A1 types derived from corals in the Indo-Pacific (Baillie et al., 2000a). Again, the results in my study support the results from Baillie et al.’s work, showing three distinct type profiles of type A1 found within clams alone. However, because my study lacks data from coral *Symbiodinium*, I can only comment on the distinct types within clams.

Due to this high diversity seen within Clade A, and more specifically type A1, it has been suggested that Clade A *Symbiodinium* most likely undergoes sexual reproduction to maintain such a diverse community within one clam host (Baillie et al., 2000b; Rodriguez-Lanetty, 2003). This idea that *Symbiodinium* may be able to sexually reproduce emerged after realizing its immense diversity (Rowan, 1998), and the increasing amount of research showing the diversity of the genus helps to support that. The diversity of *Symbiodinium* types shown in my study supports this
idea as well. Although the differences in the ITS2 gene region are only a few nucleotide base pair differences, that is enough to indicate different types, (Baillie et al., 2000a; LaJeunesse et al., 2010; 2014). Further research of diversity of *Symbiodinium* may resolve the classification of this genus to be more on par with families or orders.

Because *T. maxima* are found in shallow waters in our sampling region, it is sensible that their *Symbiodinium* type would be specially adapted for warmer waters and higher irradiance levels. Type A1 from our bleached clam samples comprised the majority of samples as the dominant type, which may support the claim that Clade A is an opportunistic clade, which was suggested by Stat et al. (2008). However, *T. maxima* in other regions that host Clade A have been found to be less tolerant of high temperatures and light levels (Sison, 2003; DeBoer et al., 2012). Also, my data shows that the type profiles A1-A1aw and A1-A1av are present in healthy clams, indicating that types within Clade A and A1 types are not always opportunistic in a parasitic sense. This could suggest that differences in types in Clade A allow for different functions, and thus, identifying the specific type profile of type A1 variants would allow for better determination of types that may be thermally tolerant or just opportunistic. Results from my study show that A1-A1aw is dominant in the inshore reef, Abu Shosha, which frequently experiences higher temperatures than the mid shelf and offshore reefs (NOAA SST data). This may indicate that the type profile A1-A1aw is thermally tolerant. However, it is difficult to support this because the clams that did harbor A1-A1aw in the bleaching
experiment did experience bleaching and not enough clams were able to fully recover to examine if there was a preference of type profile taken up post-bleaching.

The giant clam was rarely found bleached on reefs in the sampling region of this study, even during the most recent bleaching event that occurred in the summer of 2015 (personal observations). Many observations of bleached corals coincided with healthy, un-bleached clams. As A1 was found in high abundance in bleached clams in the bleaching experiment, A1 may be an opportunistic type as described in a previous study in corals (LaJeunesse et al., 2015) only able to dominate in the animal host when the previous types can no longer tolerate the increase in temperature. This supports the results of Robinson and Warner (2006), which indicate that type A1 is thermally tolerant but not suitable for ideal host growth. In my study, clams that completely recovered then shifted from A1 to A1-A1aw or A1-A1av dominance, which is similar to how corals shift back to their pre-bleached symbiont community (Toller et al., 2001; Thornhill et al., 2006). To support these conclusions further, future work on type A1 Symbiodinium and its relationship with healthy and bleached hosts could aid in the understanding of its benefit to the host. Although the characterization of opportunistic may be supported by the type A1 dominance in the bleached clams, we cannot conclude that the entire Clade A is opportunistic.

As it is clear that T. maxima associates with type A1 and its variants as its dominant type and shifts are only seen within this type, it is possible that Symbiodinium type and ability to shift are not the main techniques that clams rely on for tolerating high temperatures. As mentioned before, giant clams are filter feeders
and rely on their heterotrophic capacity as a food source with some species of tridacnids relying less on their algal symbionts for food and energy (Jantzen et al., 2008). Those species may be better off during bleaching events if they are able to acquire their own food source efficiently. Previous studies have found that corals that have higher heterotrophic capacity and that are provided extra nutrients do not bleach when exposed to bleaching-inducing temperatures in aquaria (see Leggat et al., 2003; Anthony et al., 2009). Perhaps the high heterotrophic capacity of clams allows them to be more heat and stress tolerant than corals. Another difference that could support why bleached clams are not seen as often as corals is that clams store their algae differently than corals. With stacks of algae in specialized tubules, *Symbiodinium* in clam tissue could require more effort to expel than the single-cell layer of *Symbiodinium* residing in corals. The tubule system may be advantageous in resistance against bleaching for giant clams (Blidberg et al., 2000), however research on the tubule structure as an advantage in times of thermal stress is something for future work, as it has not been described.

It is true though that these photosymbiotic holobionts evolved this symbiotic relationship that is essential for their survival. Although bleached clams are not as common to see as bleached corals from my own observations, the effects seen in the bleaching experiment in this study suggest that giant clam bleaching will occur to a similar degree as coral bleaching when there is a long period of high temperatures. With natural temperatures in the Red Sea already reaching and exceeding 32°C in the summer (Roik et al., 2016), it is surprising that we do not see more bleached clams. However, the bleaching threshold calculated as 32°C was specific for clams in
aquaria. Most likely, these clams were exposed to additional stresses not seen on the reef, which could have induced bleaching sooner than what would be seen in the field. The lack of research on induced bleaching of giant clams makes it difficult to compare results of my study. However, induced bleaching done by Leggat et al. (2003) had a total of 24 tridacnids during their experiment of eight weeks and only saw three mortalities after bleaching. However, within their experimental design, they added cultured Symbiodinium and nutrients to their treatment tanks, which most likely increased the likelihood of survival during thermal manipulations (Leggat et al., 2003). Another difference was that their study was only eight weeks while the present study was over the course of five months. In the same study done by Leggat et al. (2003) they observed naturally bleached clams and corals after a large beaching event in Orpheus Island (Northeastern Australia) and determined that the recovery rates for clams was much higher than that of corals, with greater than 95% recovery of giant clams under natural conditions (Leggat et al., 2003). In my study, clams were able to either partially or completely recover from bleaching, which was determined by regaining of coloration. However, recovery was quickly possible due to the large decrease in temperature (from 32°C to 27°C) over a few days, something not likely to happen in the field. Recovery may be possible for bleached tridacnids as seen in the in situ observations of Leggat et al. (2003) if temperatures do not remain above the bleaching threshold for too long. Recovery rate of tridacnids is hard to quantify due to the small amount of experiments and in situ observations. However, my personal observations and calculation of a higher
bleaching threshold than corals support the claim that tridacnids are fitter than corals during bleaching events.

The bleaching threshold defined by NOAA to be 1°C above the maximum monthly mean has been calculated based on the coral holobiont. Two different approaches with calculations of DHW and the bleaching threshold of Red Sea tridacnids have been explained in this study. By maintaining the same DHW at which we can expect significant bleaching, the bleaching threshold for clams was determined to be 32°C, which is 1°C above the presumed bleaching threshold of corals of the same region. This first approach in calculating the bleaching threshold is an accurate approach due to the fact that clams and corals have different bleaching thresholds and DHW is a measurement that indicates the accumulated thermal stress experienced by an organism. It should be noted that 1°C is a large temperature difference for photosymbiotic organisms, and therefore a bleaching threshold of 32°C for clams is much greater than that of corals. Keeping the bleaching threshold the same for clams as it is for corals of this region then shows bleaching for clams occurs at 15°C-heating weeks. The second approach of calculating DHW based on a conserved bleaching threshold allows for easier prediction of tridacnid bleaching when informed by NOAA bleaching prediction measurements. Again, these measurements have been optimized for corals and may not as accurately predict bleaching in clams. More SST data and data on clam bleaching observations in the field will allow for better calculations of bleaching measurements for tridacnids. Furthermore, the measurements for bleaching predictions provided by NOAA may not even be accurate for corals. Due to the
heterogeneity of the ocean and more specifically, coral reefs, one standard of bleaching will not be adequate for every region. It has been predicted that corals in Micronesia will have to adapt faster to warming temperatures than corals in other regions, indicating that the bleaching threshold and NOAA bleaching predictions may not be accurate for all regions (Donner et al., 2005). Due to the naturally high temperatures and the quick increasing of temperatures in the Red Sea (Chaidez et al., 2017), the bleaching threshold may be lower than 2°C above the maximum monthly mean (MMM already reaches approximately 30°C in the Red Sea). The specificity of regional bleaching requirements needs to be taken into consideration when predicting the next bleaching event in the Red Sea.

The mortalities seen on the sampling reefs over the year show that the marked giant clams were most likely not dying due to bleaching (since none was observed), but due to other causes. Potential causes of death would be sampling stress, predation, and wave energy, as these were the most likely causes to happen during the sampling year. Sampling stress did cause mortalities in the bleaching experiment. The two clams that died during the acclimation period were not exposed to above-ambient temperatures, but their byssal threads were severely damaged (from the removal from the reef) and the mantle tissue samples taken from these two individuals were larger than the usual size (2-3 cm²). This type of stress due to sampling would likely cause mortalities in the field as well. While sampling, the clam is vulnerable to predators as tissue is being cut and the shells are being kept open. As many clams were found with their shell intact but their tissue gone, predation could be a probable cause in at least half of the observed mortalities
on the reef. Predation could cause mortality in itself, but since these clams are able to shut completely and require force to open, predation probably was not the sole cause of the mortalities. More observational data on tridacnid predation is needed to support these conclusions.

Giant clams are able to attach themselves to the reef with their strong byssal threads that serve as permanent holdfasts to the reef (Munro, 1993), however, a clam can be removed with enough force, especially when the threads have been compromised. With the excess sampling stress and the high wave energy on at least two of the sampling reefs, the missing clams were likely removed from the reef. The missing clams were considered mortalities since they could no longer be sampled.

In my previous work describing the species of giant clams that live in this region, I observed a higher abundance of clams on reefs that were protected from high wave energy, which indicates that wave energy is a probable threat to giant clams.

In reality, although bleaching of giant clams will increase in the future with rising SSTs, other causes will likely lead to giant clam decrease. Considered a delicacy in many countries and a common species in aquariums, the giant clam’s largest threat is human harvesting (Richter et al., 2008; Copland & Lucas, 1988; Lucas, 1994). In response to the decline in tridacnid clams, all eleven species have been protected under the Appendix II of the Convention of International Trade in Endangered Species of Wild Fauna and Flora (CITES; UNEP-WCMC, 2007) and listed on the IUCN Red List of Threatened Species (Mollusk Specialist Group, 1996).

The results from the symbiont shifting in giant clams can be applied to climate change research for coral reefs. Studying the giant clam holobiont proves to
be useful because it hosts similar *Symbiodinium* types as corals (Rowan et al., 2006), it suffers from the same threats due to climate change, such as bleaching (See Norton et al., 1995; Addessi, 2001; Leggat et al., 2003; Krishnan et al., 2011), they supply large amounts of host tissue and associated zooxanthellae, and they are potentially sensitive indicators of disturbances in the environment (see Belda-Baillie et al., 1998). Knowing that Red Sea tridacnids harbor A1 type profiles as their dominant type and that these types are never present as background types, can aid in the understanding of coral type profile dominance and if their shifts to different type profiles come from those previously existing as background types. From my data, it cannot be concluded that type shifts are derived from proliferation of background types that outcompete the weaker dominant type since all type profiles were never present in the background communities. This could suggest the ability of tridacnids to take up *Symbiodinium* types from the environment. If this is possible in clams, it could be possible in corals, giving corals a better chance of surviving during bleaching events if a stress tolerant type is available from the environment. Furthermore, with the application of the SymPortal analysis and the detail of the diversity within clades of *Symbiodinium*, both ecologically and genetically, predictions of thermally tolerant types may allow us to identify the potential for corals to fight climate change. Also, understanding that these holobionts have limits to their shifting capabilities is important in predicting their adaptability. Since *Symbiodinium* have a shorter generation time than the animal host, it is thought that quick adaptation and evolution influenced by increasing temperatures will come from the algal partner in this relationship. With more
research on the association of symbiont types within hosts living in the most extreme conditions, perhaps research can be better directed to find the types that will be able to evolve. *Tridacna maxima* clams living in shallow depths in the Red Sea are exposed to high temperatures and irradiance levels similar those predicted for other reefs in the near future (ref). Therefore, the associated *Symbiodinium* type, the holobiont’s ability to shift types, and the patterns seen in this shifting should not be overlooked. There is potential to use this holobiont study as a model for examining physiological responses of other holobionts to thermal stress with experimental manipulation and possibly clade specific symbiont integration. With *Symbiodinium* as the main focus in coral reef climate change research, identifying potential types best suited for the future is a major goal. This study directly contributes to this goal and provides essential knowledge for future research.


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Figure 1. Total abundance of sequences found in all samples processed by SymPortal. Colors represent sequences that belong to described species.
Figure 2. Bar graph showing *Symbiodinium* type proportions for seasonal tridacnid samples by season.

Figure 3. Bar graph showing *Symbiodinium* type proportion for seasonal tridacnid sampling by reef type.
Figure 4. Bar graph showing *Symbiodinium* type proportions of experimental bleaching samples by bleaching status.