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Lipid content in two *Montastrea* species of coral in the Florida Keys after the 1998 El Niño bleaching event

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Abstract

Coral reefs are some of the most sensitive ecosystems to environmental change. With massive declines reported since the 1980s, there are many concerns that they could disappear due to human activities. The most common stress response in corals is bleaching, the breakdown of the symbiosis, which leaves the coral without their main energy source: their symbiotic algae. Without enough energy, corals cannot afford to invest in reef accretion, which creates the basic habitat for reef ecosystems. Differential bleaching severity and mortality has been largely documented, with high temperatures being the main stress. During bleaching, some corals switch symbiont types to a more thermo-tolerant clade, although this might present a metabolic cost. Thermo-tolerant symbionts have been reported as opportunistic generalists, suboptimal to most coral species. A way to assess coral fitness is through the monitoring of physiological parameters such as the lipid content. In this study, lipid content was analyzed in coral tissue samples from *Montastrea annularis* and *Montastrea faveolata* collected seasonally in the years 2000-2002 following the 1997-98 El Niño catastrophic bleaching in the Florida Keys. Seasonal variation was observed in most samples. Recovery was visible but slowed down after 2001, and the probable causes for that are discussed. Lipid content was significantly correlated to physiological parameters related to the symbiotic algae, confirming zooxanthellae's role in the supply of lipids to the coral. *M. annularis* presented about half the lipids per unit surface, and we suggest that this is due to the many different types of *Symbiodinium* it contained in contrast to *M. faveolata* which presented only one type.

Key words: bleaching, Caribbean, coral physiology, lipids, *Montastrea*.

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Chapter 1 Introduction

1.1- Importance of coral reef ecosystems and current situation

Coral reefs, covering just less than one tenth of the ocean floor, are one of the most biologically diverse ecosystems in our planet and contain a number of species which is greater than in any other shallow-water marine ecosystem (Spalding *et al*., 2001). The structural complexity created by reef building corals –also called Scleractinian or stony corals- through calcification provides habitat for a huge amount of organisms that live and thrive, spawn, breed, feed and grow within coral reefs. In fact, 32 out of the 34 recognized animal Phyla are found on coral reefs, compared to only nine Phyla in tropical rainforests (Wilkinson 2002). Over 25% of the world's fish biodiversity, or more than 4,000 species of fish (Spalding *et al*., 2001), together with a number of macrofaunal species such as sharks or sea turtles and countless invertebrates (including over 800 hard coral species) are associated with coral reefs. They either spend their entire life cycles in them or use them for particular ecological functions such as hunting and foraging grounds, nursery grounds for juveniles or spawning grounds for adults.

Humans benefit from coral reefs through the array of goods and services they provide. One estimate (Cesar *et al*., 2003) gave the total net benefit of the world's coral reef ecosystems to be $29.8 billion/year. More than 450 million people live within 60 kilometers of these ecosystems, with the majority directly or indirectly depending on food and income from them through fisheries, dive tourism and coastal protection among others (Pendleton, 1995). In many cases, they are the basic protein source for island nations and coastal societies not to mention the invaluable regulating ecological services they provide in otherwise very unproductive regions. Figure 1 represents the levels of dependence of countries on coral reefs and their associated ecological services.

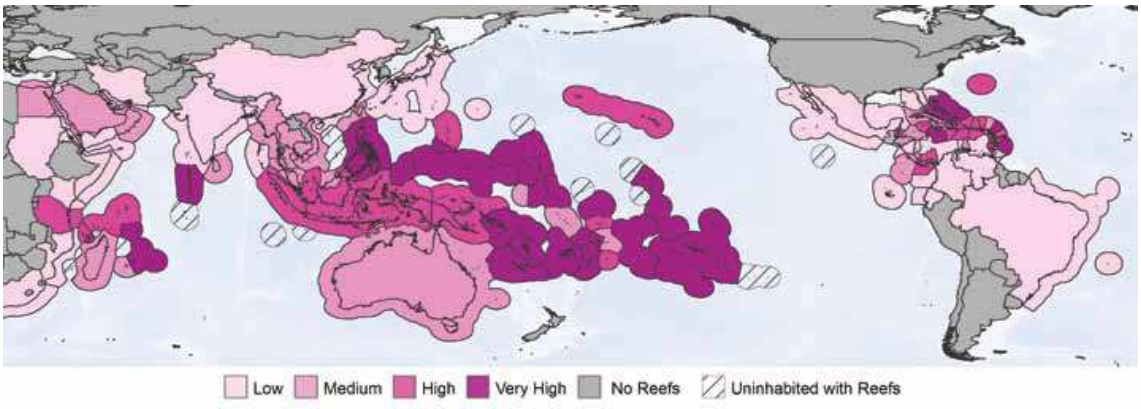

Figure 1- Reef dependence, based on reef-associated population, reef fisheries employment, nutritional dependence on fish and seafood, reef-associated export value, reef tourism, and shoreline protection from reefs (from Burke *et al*., 2011).

However, these ecosystems have experienced drastic declines over the past few decades, and 75% of the coral reefs of the world are currently degraded to some extent (Burke *et al.* 2011). Coral reefs are in decline because of anomalously warm sea temperatures associated to climate change, which are driving an increased frequency of coral bleaching and mortality (Hoegh-Guldberg *et al*, 1999), and new disease outbreaks due to host range shifts (Harvell *et al*, 1999). This in turn results in a loss of architectural complexity or flattening of the reefs, as corals are not able to keep renewing them through reef accretion. On top of that, local impacts such as nutrient input, pesticides, heavy metals or pathogens brought in by polluted run-off, overfishing, and habitat destruction, are responsible for weakening and breaking ecological linkages, promoting the appearance and proliferation of diseases and seriously impairing ecosystem resilience or its ability to recover from natural and anthropogenic disturbances (Hughes *et al*., 2003). As a result of all these pressures, complex coral-dominated reef ecosystems are likely to be rare by 2050 (Hoegh-Guldberg *et al.*, 2010). Figure 2 shows a map of the local threats on the different reef systems of the world. It is

important to note that this representation excludes the increasingly important global threats, hence being an underestimation of the potential situation of coral reefs.

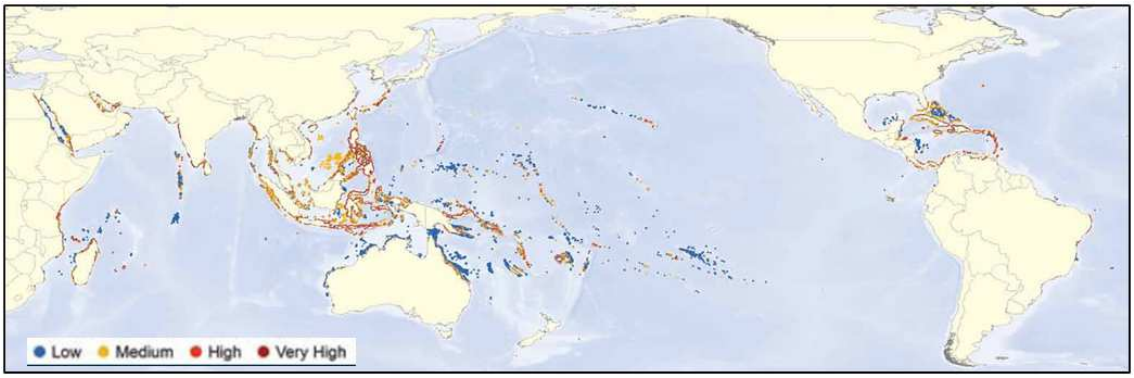

Figure 2- Reefs at Risk Revisited (2011) Coral reefs of the world classified by integrated local threat level. The index combines the threat from the local activities (1) fishing, (2) coastal development, (3) watershed-based pollution and (4) marine-based pollution and damage (Burke *et al.*, 2011).

Bleaching occurs when corals lose their photosynthetic microalgal endosymbionts and/or their pigments, and it is usually associated with El Niño Southern Oscillation events and climate change (Hoegh-Guldberg *et al*, 1999) as they drive protracted periods of warm temperatures which the algae cannot withstand. Since corals receive most of their energy from their symbionts (Muscatine *et al.*, 1981), losing them implies a period of starvation or disease which may end up in death unless they regain a large enough number of zooxanthellae before exhausting their energy reserves (Fitt *et al.*, 2001). Bleaching is typically triggered by elevated seawater temperatures and increased UV radiation (Wilkinson, 2000), but there are other stressors suspected of producing this response from corals, such as sedimentation, freshwater dilution, excess nutrient

input, and diseases (Glynn, 1996). So far, bleaching (and possibly disease) is the main indicator used to estimate coral stress up to now. However, bleaching severity and mortality has been shown to vary among coral colonies and species, and even colonies of the same species in the same site were observed to be differentially affected during the same bleaching event (Fitt *et al*, 1993). This phenomenon highlights the gap of knowledge which exists regarding a detailed understanding of the organismal responses involved in bleaching.

The picture for corals is grim. With the alarming increase in human population in coastal areas, local stressors are being exacerbated. Corals bleach and lose tissue biomass and therefore their resilience to cope with further stressing conditions, as they must rely in energy reserves. Climate change, added on top of that, is driving high temperature events that occur more often and last longer, increased storminess, increased hypoxic zones, and ocean acidification which reduces the ability of corals to produce their calcium carbonate skeleton. However, the archeological record suggests that Scleractinian corals have been around for millions of years (Stanley Jr., 2003), going through similarly stressful environmental conditions, but on a much extended time scale. If so, corals must have mechanisms that allow them to cope with stress, concept which triggered the formulation of the controversial Adaptive Bleaching Hypothesis (Buddemeier and Fautin, 1993). It has been shown that different clades of the *Symbiodinium* algae have different degrees of tolerance to temperature, which allow them to resist long periods of high water temperature (Berkelsman & Van Oppen, 2006). Although it was initially seen as a nut of hope for the survival of corals, evidence shows that there are trade-offs to surviving bleaching through increasing thermo-tolerant symbiont concentrations (reviewed in Stat & Gates, 2011), given that most

corals show specifity in their symbiosis (LaJeunesse *et al*., 2004), other symbionts not being so functionally effective for a particular coral as the specific ones. Studying the physiological changes in coral tissue during stressful events and through time is essential to understand how the symbiosis works, and how it is affected by such events occurring in protracted periods of time.

In order to understand how climate change will affect these ecosystems it is necessary to start small and scale up, starting by understanding how the symbiosis works and the ways both the coral and the zooxanthellae, individually and combined, respond to stress. Recent reviews (Knowlton *et al.*, 2008; Sotka *et al.*, 2005) remark that understanding the physiological or ecological mechanisms involved the vulnerability of corals to climate change, and a deeper knowledge of the basic biology underlying coral-symbiont interactions are essential future steps. For that, there is a need for monitoring and studying the evolution of physiological parameters and health status of corals through time, which will allow elucidating what are the "normal" and "abnormal" patterns in such indicators.

1.2- The coral-algal symbiosis

Scleractinian or reef building corals establish a mutualistic relationship with dinoflagellate endosymbionts from the genus *Symbiodinium*, commonly known as zooxanthellae, which are photosynthetic microalgae located in vacuoles of the coral's gastrodermal cells (Wakefield *et al*., 2000). This association is commonly known as the holosymbiont or symbiosome. Through this relationship, dinoflagellates receive nutrients from the coral's waste products, have a beneficial position in the water column to obtain sunlight and are better protected from grazers. In return, they produce and

translocate metabolic compounds such as sugars, aminoacids and lipids to the host (Trench, 1971), providing up to 100% of its daily metabolic requirements (Muscatine *et al.*, 1981). Although corals can also acquire carbon heterotrophically by feeding on zooplankton (Porter, 1974) or digesting particulate organic matter (Anthony, 1999) captured by the polyps, in general they rely mostly on the energy and compounds translocated from the zooxanthellae. This is the reason why the highly productive coral reef ecosystems are found in otherwise very unproductive waters (Muscatine & Porter, 1977).

Zooxanthellae can be acquired by maternal inheritance (vertically) or, more commonly, anew with each generation from the surrounding seawater (horizontally), when they must invade their host and form a functional partnership in order to persist (reviewed in Davy *et al*., 2012). There are nine divergent lineages or clades in the genus *Symbiodinium*, named A-H, and each of them contains many subclade types based on rDNA (reviewed by Stat *et al*., 2006). These are functionally distinct evolutionary entities designed with alpha-numeric names, and are the equivalent of the species classification (LaJeunesse *et al.,* 2010). This genetic diversity is likely to correlate with an equally diverse range of physiological properties in the host-symbiont assemblages, some showing greater temperature resistance (typically clade D), and some triggering faster growth rates in juvenile coral hosts (Stat *et al*., 2008). Furthermore, symbiont types explain and define niche specialization and physical distribution in many corals (e.g. Iglesias-Prieto *et al*., 2004). Corals can switch symbiont types if the environmental conditions change, provided that different clades are present. However, stability and specificity has been observed in associations between coral species and symbiont types, especially in reefs less exposed to environmental stress (LaJeunesse *et al*., 2004). In

fact, it is likely that each coral species has naturally evolved with one or a few optimal clades which make a more physiologically beneficial association.

1.3- Corals' response to stress: bleaching, switching symbionts, and heterotrophy

Beginning in the 1980s, the frequency and widespread distribution of reported coral reef bleaching events has increased (Glynn, 1996). Bleaching, or the paling of the coral, usually occurs as a result of decreasing the number of symbiotic algae in coral tissues from zooxanthellae death *in hospite*, host cell apoptosis or necrosis, or sloughing of host cells, among others (Gates, 1992). It can also happen as a result of reduced photosynthetic pigment production by zooxanthellae under stressful conditions (Kleppel *et al.*, 1989), typically when water temperature elevates beyond the tolerance threshold of the coral-*Symbiodinium* symbiosis for a given region (Porter *et al*., 1989).

When corals bleach and rapidly lose their native symbiont population, there are a few mechanisms by which they may respond (reviewed in Gates & Edmunds, 1999). One of these is by increasing heterotrophy as an alternative energy source (Grottoli *et al*., 2006). Another relevant response is corals' ability to re-populate their tissues with a species of symbiont that is more tolerant to thermal and/or light stress (Buddemeier *et al*., 1993). This is done in two ways: (1) by "shuffling"- i.e. advantageous growth of a background resident population-, or (2) by "switching"- i.e. uptaking a new symbiont from the environment- (Berkelmans & van Oppen, 2006). These thermo-tolerant symbionts typically belong to clade D, and specifically species D1a or *Symbiodinium trenchi*, as named in LaJeunesse *et al.* (2005). However, research on their biogeographic distribution (reviewed by Stat & Gates, 2011) reveals that they are uncommon in

healthy reefs and only found in higher abundances on those exposed to local stressors or with a history of bleaching. This pandemic-like distribution suggests that they may actually be opportunistic and generalists, only able to outcompete more thermo-sensitive clades in health-compromised corals, but producing a suboptimal relationship otherwise. Furthermore, some authors have categorized such association as parasitic (Stat *et al.*, 2008) rather than symbiotic or mutualistic. The genetic identity of the zooxanthellae has been shown to influence the overall performance of the coral holobiont, as different clades seem to have different evolutionary trajectories. Corals that survived bleaching show a reduction in reproductive capacity, growth rates and resistance to disease (Bruno *et al.*, 2007), and it is possible that this is due to a less physiologically beneficial symbiosis. If that is the case, corals may not deteriorate as quickly as initially expected, but changing symbiont types may just be one more phase before the death of a coral.

1.4- Lipids and their potential as bioindicators of coral status

Corals tissues contain a high proportion of lipids, and values between 9 to 47% of dry weight have been reported (Harland *et al.*, 1993). They are mainly originated from excess photosynthetically fixed carbon that is stored in the host tissue as lipids (Patton *et al.*, 1977). Therefore, lipid levels are closely linked to zooxanthellae activity within the host cells, and thus show variation depending on light intensity and water temperature (and therefore with depth). For instance, Harland *et al.* (1992) found that storage lipids increased when corals were exposed to higher proportions of light level, Oku *et al.* (2003) found that lipid content correlated both with light intensity and sea

surface temperature, and lipid-bodies within corals have been shown to vary even on a daily basis according to the amount of solar irradiation (Chen *et al.*, 2012).

Lipids can be classified according to their function in the organism. Polar lipids such as phospholipids and sterols have structural function forming cell walls and membranes, and non-polar lipids such as wax esters and triacylglycerols are storage lipids and represent significant energy reserves (Harland *et al*., 1993). This is of special relevance in case of bleaching because the primary role of lipids is to serve as long-term energy reserves (Grottoli *et al*., 2004). Indeed, Grottoli *et al*. (2004) and Yamashiro *et al.* (2005) found a lower proportion of triacylglycerols and wax esters in bleached colonies of two species of coral, suggesting that their reliance on those compounds when their symbiotic algae stop or slow down their photosynthetic activity. Lipid saturation in the thylakoid membranes of zooxanthellae has also been shown to define the algal thermal tolerance, being a potential diagnostic of the differential nature of thermally induced bleaching (Tchernov *et al*., 2004).

Physiological parameters such as tissue biomass, photosynthetic activity and algal concentrations vary seasonally (Fitt *et al*., 2000; Thornhill *et al.*, 2011). Lipid content shows seasonal fluctuation too, which is consistent with the fact that most animals tend to store more lipids in one season. However, this variation does not occur at the same time across geographic locations. For instance, in Caribbean reefs higher values occur in winter and spring (Figure 3), while lower values happen at the end of the summer and fall (Fitt *et al*., 2000). In Hawaiian and Japanese corals, however, an opposite trend has been reported (Stimson, 1987; Oku *et al*., 2003). Since lipid levels have been correlated both with light intensity and water temperature, those differences could be caused by the combination of both parameters. In fact, a number of other features such as morphological differences between coral species, such as colony morphology, tissue

thickness and polyp size (Alamaru *et al.*, 2009) can be involved in lipid utilization by corals. Looking at lipid utilization allows examining the host's response to bleaching, which may or may not be the same to that of the zooxanthellae, because about 90% of the coral lipids pertain to the animal-host fraction (Patton *et al.*, 1977). It can also provide insight on the stress-response physiological mechanisms and recovery strategies used by different species. A relevant coping strategy is to increase the amount of carbon acquired heterotrophically by the coral host. Dramatic increases in CHAR (Contribution of Heterotrophically acquired carbon to Animal Respiration) have been reported in *Montipora capitata* in bleached and recovering corals (Grottoli *et al.*, 2006), and that heterotrophic carbon is used for tissue building during recovery (Hughes *et al*, 2010). Furthermore, *Montipora capitata* recovered much faster than *Porites compressa* in artificial bleaching experiments even though it showed a lower tolerance to increased temperature, suggesting different coping strategies (Rodrigues & Grottoli, 2007). Species without heterotrophic plasticity may be able to withstand stress conditions for longer periods of time, since they cannot replenish their energy reserves once they lose their photosynthetic source. Bleached *Montipora verrucosa* did not show significant changes in total lipids in opposition to *Porites compressa*, and the study (Grottoli *et al*, 2004) proposed two explanations: either it re-allocated lipid resources from non-bleached to bleached portions of the colony, and/or has a lower metabolic rate which allows for the conservation of lipids irrespective of bleaching condition. Using $\delta^{13}C$ isotopic analyses, Almaru *et al.* (2009) showed that a species changed its carbon source for lipid synthesis from an autotrophic to a heterotrophic source linearly with depth, while another species used heterotrophically acquired carbon regardless of depth. However, other studies have shown that there are species which remain fully autotrophic under stress conditions (Edmunds & Spencer Davies, 1989). Finally, despite

triacylglycerols and wax esters are typically depleted during bleaching episodes, other studies suggest that different corals may present differential lipid class depletion (Grottoli *et al*, 2004).

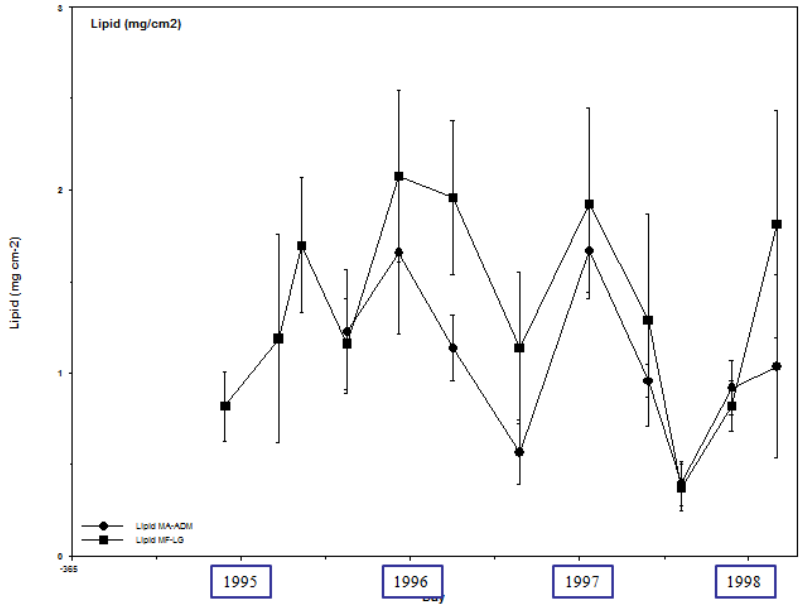

Figure 3- Seasonal lipid content in *Montastrea faveolata* and *Montastrea annularis* colonies of the two reefs studied in this thesis, Little Grecian and Admiral reef respectively, during years 1995-1998 (provided by W. Fitt, unpublished data).

In natural systems, corals seem to recover their chlorophyll *a* (chl *a*) levels and algal symbiont concentrations within a couple of months to a year after bleaching, while tissue biomass and energy reserves take longer to reach pre-bleaching levels, typically more than a year, even if chl *a* and algal concentrations are normal (Fitt *et al.*, 2000). Since lipids are directly dependent on zooxanthellae productivity, it is expected that corals with different *Symbiodinium* clades show a differential recovery after bleaching.

Cooper *et al.* (2011) showed that clade D types of *Symbiodinium* had more relative lipids in shallow water than C types, contrary to the expected. These findings might have to do with using the relative fraction ratio instead of absolute amount of lipids, but they may also be caused by the fact that corals harboring clade D *Symbiodinium* are subjected to less thermal stress.

1.5- Lipids in two *Montastrea* species in the Florida Keys: hypotheses of the study

The northern Florida Keys (USA) are subjected to large seasonal variations in temperature, typically ranging from 20-31ºC. As they live in their latitudinal threshold, coral reefs in the area have been seasonally subjected to temperature stress. For instance, the El Niño Southern Oscillation (ENSO) triggered massive bleaching events in 1982, 87-88, 97-98, and 2005, and a massive dye-off was reported in 2010 in Admiral Reef (one of the sites studied in this thesis) when extremely low temperatures were reported for more than 2 weeks (Kemp *et al.*, 2011). El Niño 97-98 led to the loss of about 16% of the world's coral reefs (Wilkinson 2000) over 42 countries, being the most widespread and severe bleaching known up to date (Fitt *et al.*, 2001).

Montastrea species (Figure 4) are boulder, structure building corals and morpho-functional species, very important for creating the complexity of the coral reef habitat. In fact, they are the most important reef-building species of the Caribbean region (Croquer & Weil, 2009). Their dominance has also been correlated with the highest reef architectural complexity in Caribbean sites, and they are therefore expected to sustain more biodiverse and functionally important ecosystems (Alvarez-Filip *et al.*, 2011).

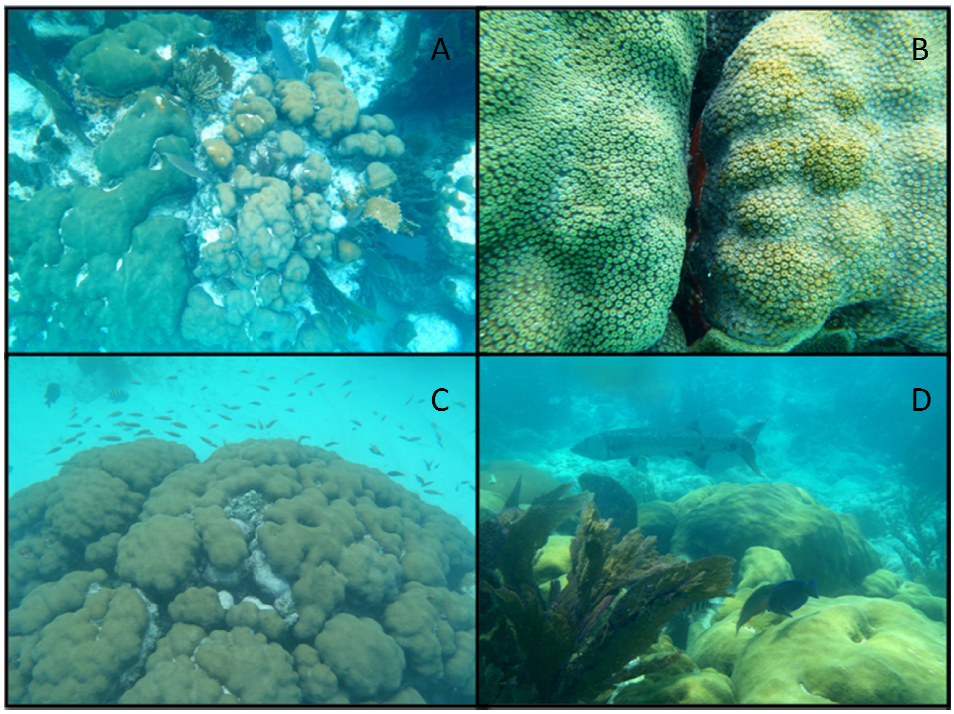

Figure 4- Pictures of the two species studied in the Upper Florida Keys. (A) *Montastrea faveolata* in the left, and *Montastrea annularis* in the right; (B) a close-up image of two different *M. faveolata* colonies; (C) and (D) *Montastrea faveolata* colonies within the reef environment.

Montastrea corals have been in decline due to a combination of the successive massive bleaching events mentioned above and disease outbreaks such as the Yellow Band Disease, triggered mostly by increasing annual mean seawater temperatures (Harvell *et al*., 2009) affecting corals when they are in the process of recovering from bleaching (Bruckner 2012). In the 2010 extreme cold temperature event, *M.faveolata* was one of the most negatively affected species. During mass bleaching events like the one triggered by the 1997-98 El Niño, *Montastrea* colonies tend to bleach in patches and survive with partial mortality (Bruckner 2012), maybe due to the fact that they show a marked plasticity in harboring different symbiont types. *Montastrea annularis* and *M.faveolata* are known to harbor more than one symbiont type, and to present different

symbiont clades during bleaching and recovering phases (Toller *et al*., 2001). Even within the same colony, Kemp *et al*. (2008) showed that there is a microhabitat distribution of *Symbiodinium* types in *M.faveolata* according to the level of exposure to light. In the case of the 1998 El Niño bleaching, Thornhill *et al.* (2006) analyzed symbiont genetic identity in samples from tagged colonies in the years following a bleaching event. As shown in Figure 5 for Admiral and Little Grecian reefs, *M. faveolata* did not have a substantial change in symbiont type from 2000 on, while *M. annularis* did harbor four different symbiont types in changing proportion. In order to tackle the question of coral lipids recovery after bleaching, total lipid content was quantified in the same tissue samples with the purpose of testing the following hypotheses:

1) *In situ* recovery after El Niño 1998 bleaching occurs over a multiyear period, with seasonal fluctuations producing higher values in winter/spring and lower in summer/fall.
2) Symbiotic algae influence on lipids: lipid content should correlate to Symbiont-related physiological factors and zooxanthellae genetic identity.

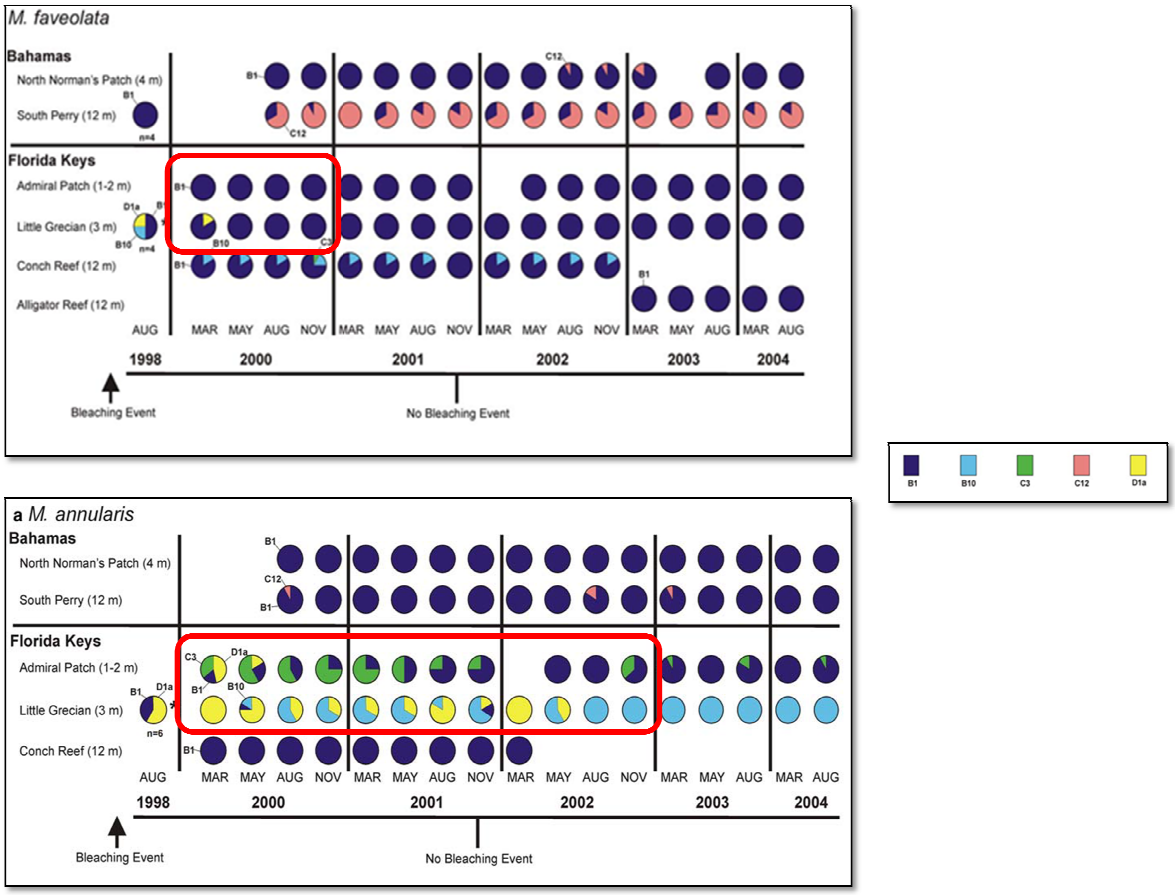

Figure 5- *Montastrea faveolata* and *Montastrea annularis* ITS 2 *Symbiodinium* types detected from the Bahamas and the Florida Keys from August 1998 to August 2004 in six colonies (from Thornhill *et al*., 2005). Of particular interest for this study are the ones highlighted in red, as they belong to the samples analyzed for total lipids.

Chapter 2 Methods

2.1- Study site and sample collection

Field collections and processing are explained in Thornhill *et al* (2005). In short, coral tissue samples of two species of scleractinian corals, *Montastrea annularis* and *Montastrea faveolata*, were collected from six targeted colonies at two sites off the Upper Florida Keys (USA), the inshore Admiral reef (1-2 m depth, 25.05ºN, 80.39ºW) and the offshore Little Grecian reef (3-4 m depth, 25.12ºN, 80.30ºW) (Figure 6). The colonies were identified and tagged to ensure the follow-up of the same individuals. Samples were collected by SCUBA each season (March, May, August and November) during the years 2000, 2001 and 2002 in the case of *Montastrea annularis*, and 2000 for *Montastrea faveolata*. Approximately 5-cm diameter tissue samples were extracted via hammer and chisel, making sure that the same relative position (i.e. the unshaded colony tops) was sampled each time. Coral fragments were placed in seawater-filled, pre-labeled plastic bags and transported immediately to the laboratory in an insulated cooler, were they were processed immediately.

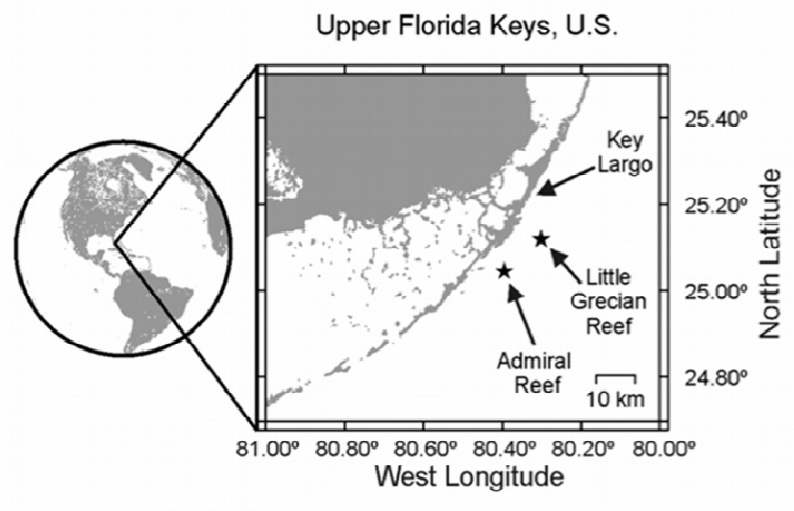

Figure 6- Geographic location of the two research sites off Key Largo in the Florida Keys, USA. Admiral reef is more shallow (1-2m) and protected, while Little Grecian is an offshore, deeper reef (3-4m).

Coral tissue was removed from 5 to 25 cm^2 of coral skeleton with a recirculating Water Pik™ using filtered (0.45µm) seawater. This salt-water blastate was pulsed briefly (1-4s) using a Brinkmann Instruments Polytron Kinematica Tissue Homogenizer™ to disperse mucopolysacharides and freeze dried. Three replicate aliquots of about 10 mg tissue were combusted to determine ash-free dry weight.

2.2- Lipid extraction

Absolute lipid content was determined by the gravimetric method. Lipids were extracted from the freeze-dried samples using a variation of the Folch method (Folch et al., 1957; complete protocol can be accessed in the appendix). In a nutshell, around 10mg of tissue sample were extracted using Chloroform-Methanol (2:1) and a salt ($CaCl_2$) and then dried under Nitrogen to proceed to the weightings. After the gravimetric determination of the lipid content, it was standardize to coral tissue surface area. A blank was made for every set of samples, adding/subtracting its value to the respective batch of samples.

It is worth mentioning that the present method of extraction does not discriminate between symbiotic algae and animal. However, it can be assumed that the measures reflect mostly the host's response because about 90% of the coral lipids pertain to the animal-host fraction (Patton *et al.*, 1977).

2.3- Data and statistical analysis

Average lipid content was calculated by averaging the weights from the three replicates of each tissue sample, and then divided by the surface area which had been

waterpiked during sample processing in the field. Sea surface temperature (SST) monthly average data for the 1º Lat x 1º Long square nearest to the study sites was obtained from the International Comprehensive Ocean-Atmosphere Data Set (ICOADS) online database (http://dss.ucar.edu/pub/coads, Slutz et al., 1985; 23.07.2012), and a seasonal average was calculated as the mean of the three months previous to the seasonal sampling, i.e. winter (Dec-Jan-Feb), spring (March-April-May), summer (June- July,-Aug), and fall (Sept-Oct-Nov), after verifying that the particular monthly means pertaining to the sampling were not correlated. Complementary physiological data (i.e. symbiont cell densities, fluorescence yield -Fv/Fm ratio-, and zooxanthellae types) for the studied samples was kindly provided by Dr. William Fitt (some of which has been published in Thornhill *et al*., 2005; 2011).

Statistical analyses were performed using SPSS and STATISTICA 7.0 software, and significance was considered when p-values < 0.05. The 6 different colonies studied did not show significant differences among them, and were consequently treated as replicates in all statistical analyses.

Before the statistical tests, the assumptions of normality and homogeneity in the data were verified by the Kolmogorov-Smirnov and Bartlett's test, respectively. A t-test was performed to determine whether the two species differed in lipid content, and a one-way ANOVA was performed to determine whether the lipid data significantly differed between the two sites. A General Linear Model (GLM) was used to test for statistical differences in each of the species among lipids in relation to season, year, zooxanthellae type, and site (year and zooxanthellae type were only tested for *M. annularis* due to limitations of *M. faveolata* sample representation). Afterwards, the post hoc Tukey test was applied to further assess differences between seasons and years. The statistical

analyses between different years could only be done on *M.annularis* data, since *M.faveolata* samples only covered one single year (2000).

Finally, the data was separated according to site, and then Pearson correlation tests were performed to evaluate the influence of each of the factors on lipid content (previous confirmation of the normality and homogeneity assumptions).

Chapter 3 Results

3.1- Patterns of lipids in relation with temperature

Figure 7 shows the calculated seasonal sea surface temperature (SST) for the area of study. Higher mean summer temperatures are clearly noticeable during the years 1997 and 1998, corresponding to El Niño events. Mean SST in summer of 2005 was higher as well, and it corresponds to massive bleaching event in the Caribbean that was not related to the ENSO (Eakin *et al*., 2010). It is worth noticing that summer of 2001 presented a mean SST higher than the rest of non-bleaching years.

Regarding low temperature extremes, the lowest mean winter SST was that of 2000. The two lowest fall temperatures occurred in bleaching years (1997 and 2005, Figure 7). Spring means were the ones presenting the most marked variability.

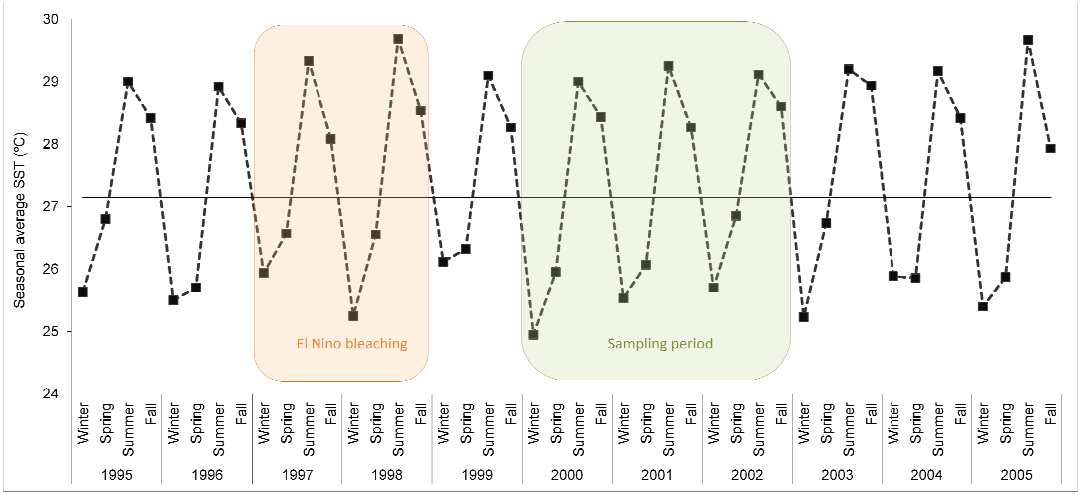

Figure 7- Seasonal mean SST between 1995 and 2005; highlighted are the period of El Niño bleaching event and the sampling period. The horizontal line represents the average SST in the area calculated from the 30-year monthly averages (1975-2005).

In Figure 8, total lipids are represented together with mean seasonal SST for the studied time period. The general visual trend suggests that lipids were lower when temperatures were higher for each three-month period, and vice versa. However, results from the GLM indicate that this does not happen in all cases as total lipids were not correlated to sea surface temperatures in the case of *M.annularis*. On the other hand, they were for *M.faveolata* ($F_{(1,43)}$= 7.410, p= 0.009) and this correlation was robust, because once separated by site, it was still significant for both Admiral reef ($F_{(1,43)}$= 9,328, p= 0.007), and Little Grecian ($F_{(1,43)}$= 8.10, p= 0.011).

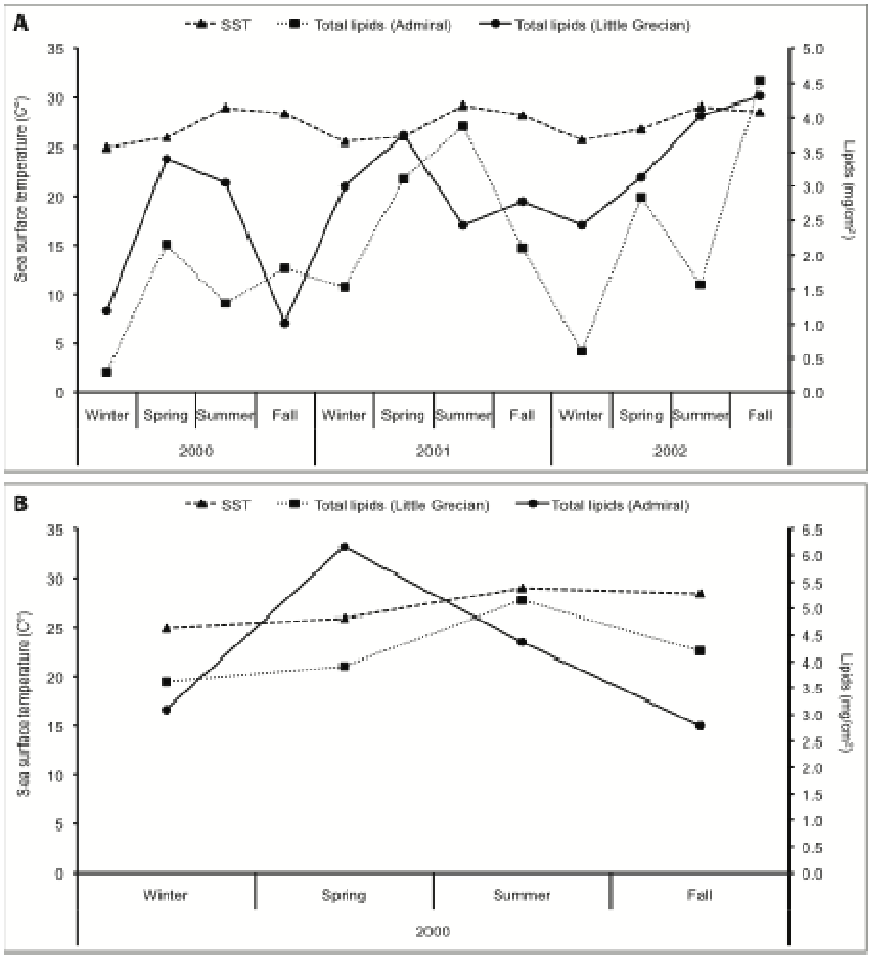

Figure 8- Total lipids in the two reefs of the study and SST over the sampling period for (A) *M. annularis* and (B) *M. faveolata*.

3.2- Patterns of lipids from different coral species

The lowest lipid content was reported for *M. annularis* in Admiral Reef in winter of 2000 (0.3048 ±0.20 mg/cm^2); the highest value belonged to *M. faveolata* in Little Grecian in spring of 2000 (6.1679 ±2.054 mg/cm^2). All the other values fell in between the two extremes. Lipid data is presented as mean ± SD throughout the entire document.

M.faveolata had about twice the lipid content of *M.annularis* (Figure 9), and the differences were significant for all seasons in the year 2000 (Table I) once they were separated by site.

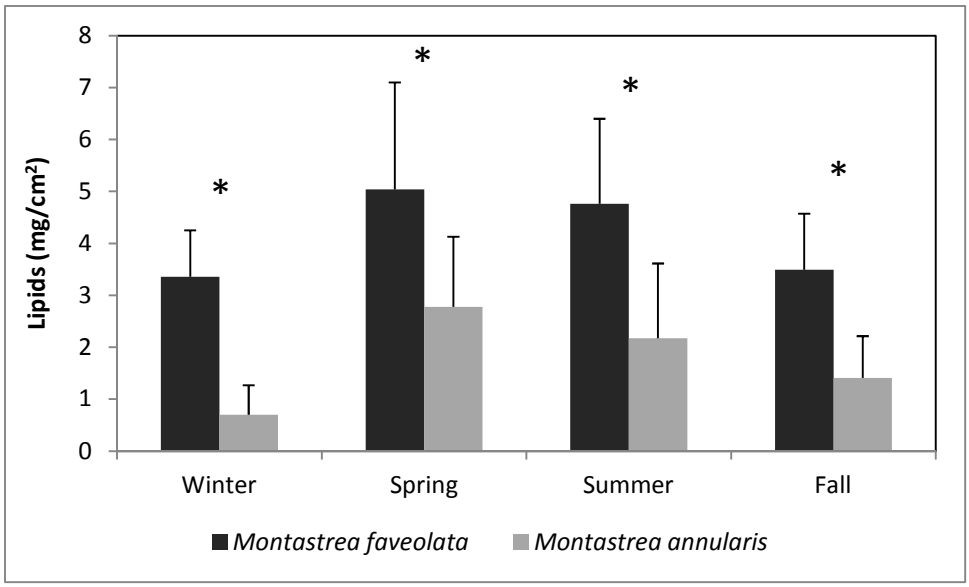

Figure 9- Average lipid content in *M. faveolata* and *M. annularis* for each season in the year 2000; (*) represent significant differences between species ($p < 0.05$).

Table I- Results of the t-tests to assess differences between species in each season.

T-test	Mean *M.faveolata*	Mean *M.annularis*	p-value	F-ratio
Winter	3.353645	0.698147	0.000000	2.486586
Spring	5.039993	2.778010	0.004292	2.336235
Summer	4.759989	2.172037	0.000462	1.294624
Fall	3.489910	1.411236	0.000022	1.811793

Both the lowest and highest lipids for *M. annularis* were reported in Admiral Reef, the lowest being those of winter 2000 (0.3048 ±0.20 mg/cm^2), and the highest during fall of 2002 (4.5395 ±1.5088 mg/cm^2). For *M. faveolata*, the two extremes were found in samples from Little Grecian Reef; the lowest value occurred in the fall of 2000 (2.7853 ± 0.5629 mg/cm^2), and the highest in spring of 2000 (6.1679 ± 2.054 mg/cm^2).

3.3- Lipid patterns over time

All factors tested in the GLM were highly significantly correlated with lipids in *M. annularis*, with p-values in the order of $p \leq 0.0001$ except for year: site ($F_{(1,119)}$= 14.087), year ($F_{(2,119)}$= 6.528, p= 0.002), and season ($F_{(3,119)}$= 6.981). However, an interaction between year and season was detected and tested together ($F_{(5,119)}$= 7.990).

The Tukey post-hoc test showed that the differences between months (seasons) were strongly significant between winter and all the others (p= 0.000), while the other seasons were not significantly different among them. Finally, the Tukey test showed that year 2000 was significantly lower than 2001 and 2002 (p= 0.000 in both cases), but those were not significantly different among them (p= 0.688). In the GLM, only season

was correlated with lipid content for *M. faveolata* ($F_{(3,43)}$= 3.932, p= 0.014), but monthly differences were later revealed by the Tukey test to be only significantly between winter and spring (p= 0.043). The year could not be tested as only one was represented.

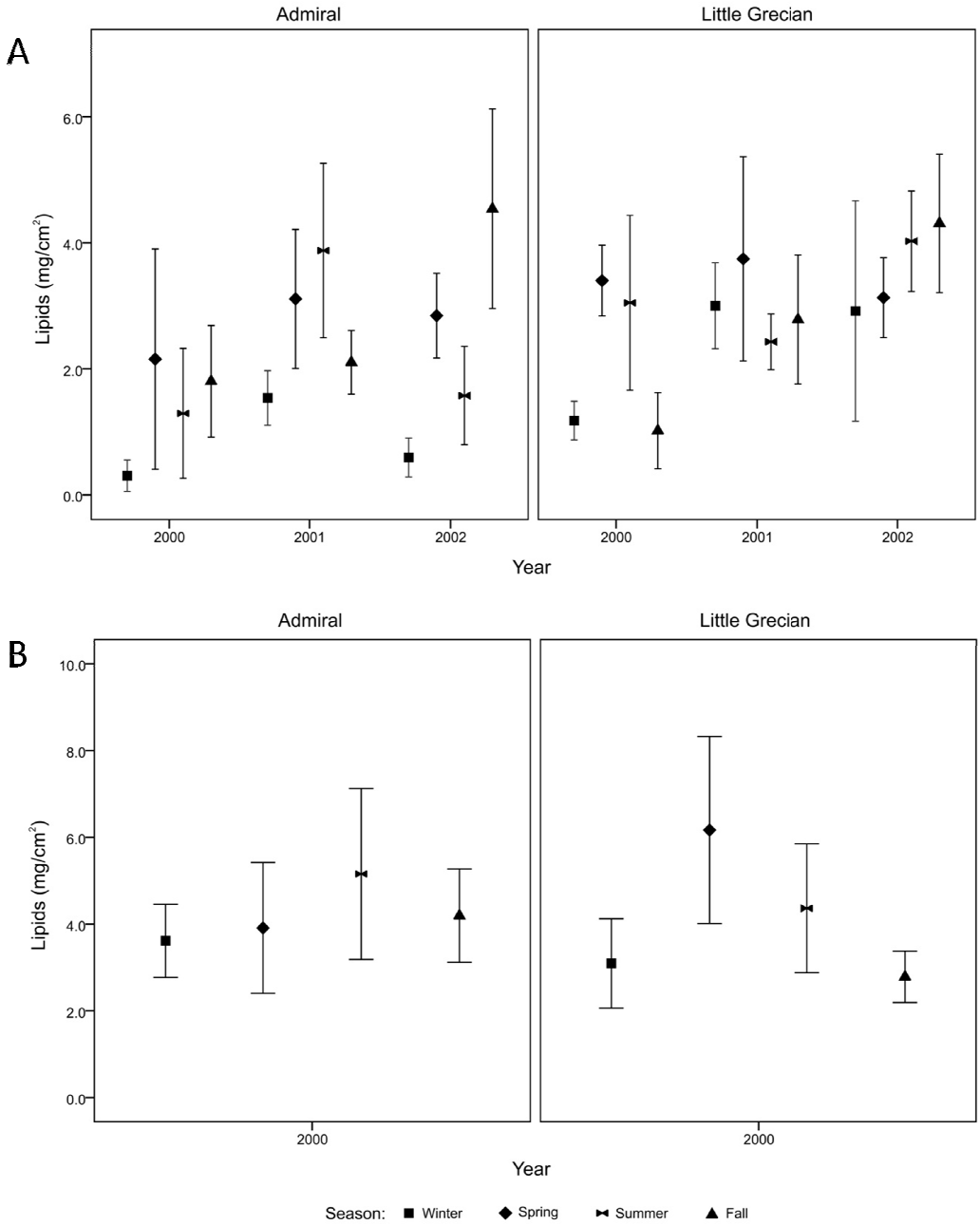

Figure 10- *M. annularis*' (a) and *M. faveolata*'s (b) lipids over time for Admiral and for Little Grecian reefs. Arrow bars represent the 95% confidence interval.

Total lipids did not show a solid seasonal pattern (Figure 10). In fact, different years showed different patterns in the case of *M. annularis*. Of particular interest are years 2001 and 2002 in Admiral and Little Grecian reefs respectively, where summer values increase instead of presenting the decrease that characterizes the rest of the years. Furthermore, for 2002 in Little Grecian lipid content does not show any fluctuation but rather a steady increase with no apparent influence of season whatsoever. *M. faveolata*'s lipids seem to be more consistent in both reefs, although data represent only one year, and differences observed in *M. annularis* are noticeable when comparing between different years. For both species, there is an increase in lipids in spring in all cases.

3.4- Patterns of lipids from different sites

Total lipids from coral tissues significantly differed between the two sites ($F= 12.50$, $p= 0.001$), and are represented in Figure 10.

At Admiral Reef, *M. annularis* lipids showed a yearly ($F_{(1,43)}= 6.607$, $p= 0.013$) and seasonal correlation ($F_{(1,43)}= 4.478$, $p= 0.039$), and seasonal for *M.faveolata* ($F_{(1,43)}= 5.945$, $p= 0.025$). Samples from Little Grecian Reef showed a correlation with year for *M. annularis* ($F_{(1,43)}= 4.487$, $p= 0.038$) but not with season, which was also found in *M.faveolata* ($F_{(1,43)}= 11.31$, $p= 0.003$). Differences in the symbiont-related physiological parameters were also found.

3.5- Lipids in relation to their symbiotic algae

In the GLM, total lipids were found to be significantly correlated to the clade of *Symbiodinium* determined for the *M. annularis* samples ($F_{(8,119)}= 2.469$, $p= 0.016$).

However, a more detailed analysis after separating the data by site yielded non-significant results ($F_{(2,41)}$= 0.261; p=0.771), largely because the presence of too many types of *Symbiodinium* and a not large enough sample size prevented an appropriate representation. *M. annularis* samples from Admiral reef were the only ones in which lipid content was significantly correlated with zooxanthellae type ($F_{(5,43)}$= 2.789, p= 0.025).

Nevertheless, the other symbiont-related physiological parameters were strongly linked to lipid content as shown by the GLM. Fluorescence yield - Fv/Fm ratio- was significantly correlated with lipids in the overall analysis ($F_{(1,119)}$= 4.30, p= 0.040), and so was zooxanthellae cell density ($F_{(1,43)}$=6.183, p= 0.017). When testing sites separately, Fv/Fm was correlated to lipid content in *M.faveolata* from Admiral Reef ($F_{(1,43)}$= 6.397, p= 0.021) and Little Grecian ($F_{(1,43)}$= 7.39, p= 0.014), and in *M. annularis* from Little Grecian ($F_{(1,43)}$= 5.633, p= 0.021), but not for *M. annularis* from Admiral Reef. Symbiont cell density was always significantly correlated with total lipids in *M. faveolata* – Admiral reef ($F_{(1,43)}$= 5.108, p= 0.036) and Little Grecian ($F_{(1,43)}$= 5.11, p= 0.036)-, but not in *M. annularis*.

Chapter 4 Discussion

4.1- Variations in lipid content with environmental conditions

4.1.1- Temperature, season and years

There was a significant recovery of lipids between the years 2000 to 2002 in the samples of coral tissue analyzed in this study. Results suggest that lipid content decreased when temperatures were higher for each three-month period, and vice versa. However, this visual trend was not confirmed statistically for *M.annularis*, mainly because seasonal patterns were not consistent throughout the years. This unpredicted trend indicates that there are other parameters affecting lipid content, either in the way it is produced or in its utilization, or both. Looking in more detail at the inconsistencies when comparing the seasonal pattern to that reported by Fitt *et al*. (2000), one can notice that the increase of lipids in summer 2001 is followed by a subsequent decrease in the fall. Also, lipid content increases in the spring rather than in winter (Fitt *et al*., 2000) for all cases despite spring seasonal mean SST presenting the most marked variability. Possibly, this might suggest a delay in the changes in lipid content as a result of being a metabolic response to environmental changes.

Corals at Little Grecian in 2002 were noticeably different because they did not show the usual seasonal fluctuation but just a steady increase in lipid content. Statistical analyses indicate that lipid content did not significantly increase between 2001 and 2002, which could be explained by the slightly higher seasonal mean SST detected in summer of 2001 as compared to the rest of non-bleaching years. Potentially, the cumulative effect of an extended period of temperature stress impaired lipid production. Besides, year 2002 had a mild winter and warmer spring and this change in the typical SST seasonal average may be the reason for the missing seasonal pattern.

M. faveolata's lipid content data seemed more consistent in both reefs, although representing only one year, and differences in seasonal trends observed in *M. annularis* are noticeable when comparing between different years. Nevertheless, *M. faveolata* lipid content was strongly correlated with SST, contrary to *M. annularis*, and this and other differences may be indicative of different lipid biological controls. Field observations and several reports indicate that *M. annularis* percent cover is conspicuously declining as a result of bleaching, disease, predation and increased competition by other benthic organisms, and that the reduced colony sizes are below the threshold for successful reproduction (reviewed in Bruckner 2012). If so, it is possible that *M. annularis'* energy allocation and lipid utilization has changed and is being invested in building and repairing coral tissue.

4.1.2- Differences in lipid content between species and sites

M.faveolata had, in average, about twice the lipid content of *M.annularis*. According to the initial hypothesis, this is indicative of the fact that *M.annularis* had changes in the type of symbiotic algae reported regularly, almost in every sampling point. This is supported by Cooper *et al*. (2011) and Jones & Berkelmans (2011), who found that different *Symbiodinium* clades significantly affect the quality of the corals' energy reserves.

Lipid content significantly differed between sites, and there was a higher variability in their response in the samples from Admiral Reef. This is probably explained by the physical location of both sites; Admiral is closer to shore, in Hawk Channel (3 miles offshore), while Little Grecian is located on the outer reef (5 miles offshore). Besides, Admiral is a shallower reef (colonies located at 1-2 meters depth vs. 3-4 meters in Little

Grecian), and is probably more exposed to short and long-term environmental and atmospheric fluctuations such as temperature, wind, waves, etc. In fact, both species had lipid levels strongly correlated with season in Admiral Reef, while only *M. annularis* correlated significantly with season at Little Grecian.

4.1.3- Symbiont-related parameters

The relationship between *Symbiodinium* type and lipid content could not be established due to a lack of sample representation. However, the other symbiont-related physiological parameters were strongly correlated with lipid content, as was expected since most of the lipids are translocated from the symbiotic algae to the coral tissues (Harland *et al*., 1993). The density of symbiotic algae was significantly correlated with lipids for *M. faveolata*, but not for *M. annularis*. Zooxanthellae density is typically correlated with tissue biomass (Fitt *et al*., 1993, Thornhill *et al*., 2011), which has been shown to be a strong indicator of coral status (Thornhill *et al*., 2011). A strong correlation was also found regarding fluorescence yield, again with the exception of *M. annularis* from Admiral Reef, indicative of the algae's photochemical capacity. Given that Yamashiro *et al*. (2005) also found that lipid content was positively correlated with zooxanthellae density in bleached Okinawan corals and that *M. annularis* showed a large variability in this study, it could be argued that this correlation is not present when switching to non-specific symbiont types.

4.2- Back to the hypotheses

4.2.1- In situ recovery after El Niño 1998 bleaching

Regarding the hypothesis that recovery of lipids after the 1997-98 bleaching event would occur over a multiyear period, it was not falsified because there was a significant increase in total lipids along the three years of the study. However, this assessment could only be done in relative terms, since there are not many studies tackling lipid recovery in corals after a bleaching event in terms of total lipids. Acceptable lipid levels for healthy corals in the Caribbean have not been determined, being in fact a challenging endeavor due to the many bleaching events the corals have been subjected to in the past decades. Fitt *et al*. (1993) sampled *M.annularis* corals at random after the 1987 bleaching event in the Florida Keys and found that lipids had significantly increased over a two year period. Yet, the surveyed colonies were not the same throughout the study period and it also lacked baseline data. Besides, results show that only the first year was significantly lower than the second and third, although a trend of increase was still noticeable. This could indicate that recovery slows down after a certain concentration of lipids is attained, potentially indicating that lipid levels may be restored in two years, or that small differences in seasonal SST did affect the recovery. Together with the fact that there is a one year lag between the bleaching event and the initial data (i.e. missing data for 1999), these results may imply that recovery takes considerably more than a year (Fitt *et al*., 2000).

Finally, seasonality was observed but with a considerable variability, suggesting that there are numerous factors driving lipid content in *Montastrea* corals. This is supported by the fact that basically all the environmental factors tested were significantly correlated to lipid content.

4.2.2- Symbiotic algae influence on lipids

Zooxanthellae genetic identity could not be correlated to lipids in this study. Nonetheless, the fact that samples containing different symbiont types were always significantly poorer in lipids than the ones pertaining to a species that did not change symbiont type after the bleaching is indicative of its important role in lipid production and/or utilization, and the recovery of storage lipids after a bleaching event (Jones & Berkelmans, 2011). The other symbiont-related physiological factors were also correlated with lipid content, confirming the importance of zooxanthellae for the supply of lipids to the host.

The coral and the zooxanthellae are affected by environmental factors; zooxanthellae are the main source of lipids, so if they are under stress, or if the coral changes to a more thermo-tolerant but less metabolically beneficial clade of *Symbiodinium,* lipid production may be reduced or halted. The coral animal, also affected by stressful environmental conditions, needs to use these energy reserves at a greater rate. These two phenomena may occur at the same time or not, but even when the stress conditions stop and bleached corals regain their zooxanthellae, the animal still needs to use energy stored in lipids to rebuild its tissue, therefore delaying full recovery of lipid levels. Moreover, even during the same bleaching Yamashiro *et al*. (2005) reported differential reduction in the lipid content in different species depending on coral morphology.

Although some species have been observed to increase their heterotrophic carbon acquisition under energy compromising conditions (Grottoli *et al*., 2006), and a marked heterotrophic behavior has been reported for the deeper-dwelling coral species *Montrastrea cavernosa* (Porter 1974) and *M. franksi* (Mills and Sebens, 2004), the species in this study appear to employ more photoautotrophic than heterotrophic

strategies to cope with environmental stress (Lesser *et al*. 2000), going back to the fundamental role of their symbiotic dinoflagellates.

4.3- Other remarks

The marked variability in the results of total lipids could be partly explained by potential errors derived from the gravimetric method, particularly the high sensitivity to small losses of mass during the analyses and the standardization to surface area. Saunders *et al*. (2005) proposed a method based on lipid functional fractions, in which the ratio of storage to structural lipids it determined by means of thin layer chromatography. This method might become a useful tool for monitoring coral health in the future, but the lack of an absolute measurement reduces considerably the information provided about coral status. The gap of knowledge regarding how different lipid classes are depleted over time and environmental conditions and which are the eco-physiological mechanisms affecting lipid formation and utilization in corals needs to be covered before this method is feasible. Clearly, bleaching and symbiont loss plays a significant role in determining resource partitioning in corals (Thorhill *et al*., 2011), but the damaging effects of thermal stress depend on the time of exposure. This has been demonstrated by lipids not being affected in short-term experimental bleaching (Fitt *et al*, 2009).

By combining a number of powerful climate models, Hoegh-Guldberg (1999) concluded that future ENSO events are likely to reach higher sea temperature thresholds and that the frequency of bleaching will rise rapidly with the rate being highest in the Caribbean. Furthermore, the same study predicts that most regions will experience bleaching conditions every year within 30-50 years. Regular exposure to bleaching will

surely devastate coral species which, like *M. annularis*, are unable to adapt to the new conditions, and the Caribbean seems a natural laboratory to document how coral reefs disappear. The order appears to be: (1) Thermal stress reducing the density of symbiotic algae in coral tissues, (2) lipid reserves being exhausted and (3) colony biomass being reduced steadily until eventual colony death (Thornhill *et al*., 2011). In fact, not only increased SST but also lower extremes are likely to occur on a more regular basis, such as the Admiral Reef catastrophic die-off reported in 2010 in which virtually all the *Montastrea* colonies died after two weeks of sustained low temperatures (Kemp *et al*., 2011). Finally, a different picture has recently been reported in some indo-pacific corals, in which even corals which are normally highly susceptible to bleaching showed rates of adaptation and survivorship in reefs that had been exposed to previous thermal stress episodes (Guest *et al*., 2012). Whether this has happened in the Caribbean region, and in what temperature ranges can adaptation or acclimation occur for different coral species has yet to be determined.

4.4- Conclusions and future directions

This study is relevant because the use of tagged colonies allowed a follow-up of the lipid content and other physiological parameters over time in the same coral, giving the chance to assess the evolution of total lipids over time after the 1997-98 bleaching event. Lipids have been regarded as a potential measurement of sub-lethal coral stress, needed in the present environmental scenario to document human and natural stressors responsible for the disappearance of corals on reefs. The very impacted Caribbean coral reef ecosystems provide the perfect testing grounds to develop such a coral health indicator. Studies on lipid content have been typically made on randomly selected coral colonies or on corals brought into the laboratory for short-term experiments (days to

weeks) without consideration to the seasonal variation in seawater temperature. For this reason, many more studies and long-term monitoring of lipids are necessary in order to establish lipid level thresholds in order to develop lipid measures as a reliable indicator of coral health. With this objective as a goal, it is of paramount importance to study the lipids of different coral species in order to elucidate the stress-coping mechanisms they use, and then understand their effect on lipid levels. This way, case-specific quantitative baselines can be established. Understanding which factors determine lipid content, and ultimately coral fitness, can inform managers to take the appropriate conservation measures to minimize the effect of global warming.

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Appendix

I. Protocol for the ash-free dry weight

1- Waterpik the coral with fresh-water (distilled water).

2- Freeze the “blastate”.

3- Lyophilize the frozen samples (= freeze dry).

4- Weigh the ppt

For each coral:

5- Take 3 replicate samples of about 10 mg (weigh each to the 0.00000g).

6- Dry them in a 60 ^{o}C oven, then reweigh.

7- Ash the sample by putting them in a 500 ^{o}C oven for at least 5h (overnight).

8- Cool, keep in a 60 ^{o}C until the final weight is obtained (weigh to the 0.00000g).

9- Ash-free dry weight is calculated form the difference between dry weight and ash weight.

II. Protocol for lipid analysis (total lipids)

Materials:

1. Chloroform : Methanol (2:1 volume), with addition of 0.2% BHT (2,6-Di-tert-butyl-4-methylphenol) to prevent lipid oxidation during analysis.

2. Folch wash (for approximately 100 ml of Folch wash). Preparation:

240 ml chloroform

120 ml methanol

90 ml 0.4% CaCl2 solution (4 g/1 L DIW)

Mix well in a separation funnel and discard the lower phase.

3. Aluminum foil, burnt in oven at 500ºC for at least 6h.

4. All tubes, pipettes and laboratory material must be from glass. No plastic can be involved in the process of extraction.

Method:

1- Weigh replicate test tubes to 0.00001 g (n = 3, three replicates per sample).
2- Weigh freeze dried samples to 0.00001 g. (~10 mg each) into preweighed test tubes, and make one blank (with no coral tissue) that will go through all the process explain below.
3- Grind each sample with 1-3 ml of Chloroform:Methanol (2:1).
4- Millipore filter and rinse well with Chloroform:Methanol.
5- Place filtrate back into original test tube and bring total volume to about 6 ml with Chloroform:Methanol.
6- Add 2 ml of the Folch wash and gently invert 3 times, removing aluminium fold cap each time to release pressure.
7- Let separate (~ 5 minutes) and then remove and discard the upper phase.
8- Repeat steps 6 and 7 one more time.
9- Evaporate under nitrogen to constant weight in an oven at 60º C. Do this step under a gas hood. Use one pipette to direct the Nitrogen flow to each tube, without touching the liquid.
10- Reweigh the test tubes to determine the lipid weight in each replicate.
11- Resuspend in 5 ml of Chloroform and keep in freezer at -40ºC for future lipid class analyses.