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Environmental stress in hard coral: Evaluating lipid as an indicator of sublethal stress on short time scales

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ENVIRONMENTAL STRESS IN HARD CORAL: EVALUATING LIPID AS AN INDICATOR OF SUB-LETHAL STRESS ON SHORT TIME SCALES

A Dissertation

Presented to

The Faculty of the School of Marine Science

The College of William and Mary in Virginia

In Partial Fulfillment

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Doctor of Philosophy

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by

David Harold Niebuhr

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APPROVAL SHEET

This dissertation is submitted in partial fulfillment of

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David Harold Niebuhr

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TABLE OF CONTENTS

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LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Lipid quality was evaluated in *Montastrea spp.* under sediment- and heat-stressed conditions. Levels of total lipid in *M. annularis* subject to experimental sedimentation were highly variable (10.07 - 210.37 mg lipid/g dry tissue) and did not differ significantly with treatment ($p > 0.25$). The ratio of storage lipid (wax ester + triacylglyceride) to structural lipid (sterol esters + phospholipid) decreased significantly (from 0.25 to 0.14, $n = 22$, $p < 0.01$) in treatment colonies. Fatty acid methyl esterization (FAME) analysis of colonies exposed to experimental sedimentation showed a reduction of the algal, 18:3(n-6) and 18:4(n-3), polyunsaturated fatty acid (PUFA) in the polar fraction of tissue lipid extracts. This loss of PUFA suggests a loss of algal membrane in sediment-stressed colonies. Lipid quality was similarly measured in 20 colonies of *M. faveolata* over a 10-day period. Mean ($n=20$) ratio of storage to structural lipid in *M. faveolata* dropped from 2.43 to a level of 0.98 ($p < 0.01$) immediately following a natural sedimentation event before recovering to levels of 1.4 and 2.9 post-storm days 2 and 4, respectively. A sensitivity analysis of lipid content with decreasing tissue sample size was conducted and stress experiments were repeated in *M. annularis* using Vacutainer® blood collection tubes to collect microtissue (3-5 polyps) samples without destroying skeleton of the sample colonies. A significant decrease in storage:structural lipid ratio (from 0.91 to 0.31, $p < 0.01$) in sediment stressed colonies was also detected using the microtissue technique. Colonies of *M. annularis* subject to heat-stress (35°C) exhibited no significant change in storage lipid ratio, while levels of Free Fatty Acids (FFA) increased significantly from 0.012 to 0.156 mg lipid/g dry tissue ($n = 22$, $p < 0.05$). FAME analysis of tissue lipids extracted from the heat-stressed colonies showed changes in the polar fraction, with significant decreases in the 18:3(n-6), 18:3(n-3), 18:4(n-3), 20:4(n-6) and 20:5(n-3) ($p < 0.05$) PUFA and subsequent significant increases in the saturated fatty acids, 16:0 and 18:0 ($p < 0.05$). Changes in lipid quantity and quality indicate possible oxidation and preferential digestion of zooxanthellar membranes in *M. annularis* subject to severe heat stress. The results of this study suggest that the relative abundance of lipid subclass components can indicate sub-lethal environmental stress in *M. annularis* and *M. faveolata* on short time scales. Results from the microtissue collection techniques are consistent with other forms of tissue analysis which require destruction of the coral colony. Furthermore, micro-tissue collection techniques permit repeated monitoring coral colonies to detect the manifestation of stress from first impact (hours/days) at the cellular level before these effects become visually apparent. This technique may be a viable method to study how individual, colony-level response to environmental stress is manifest in community structure over longer time scales (years/decades).

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ENVIRONMENTAL STRESS AND HARD CORALS: EVALUATING LIPID AS AN INDICATOR OF SUBLETHAL STRESS ON SHORT TIME SCALES

CHAPTER I

GENERAL INTRODUCTION

It is widely accepted that coral reefs develop under a limited range of conditions. These environmental conditions can be characterized as: temperatures between 18°C - 33°C; salinity in the range 27-38 ‰; high water energies (limited by shear stress); low suspended sediments, and depth limited by light availability. These baseline environmental characteristics have been well documented, in both field and laboratory studies, since the last century (Mayer, 1918, Edmondson, 1929, Yonge, 1930, Marshall and Orr, 1931, Umbgrove, 1947, Goreau, 1959, Dodge et al, 1974). While coral species have adapted to life at the extremes of these conditions (i.e. high latitude conditions vs. tropical conditions) for all parameters, it seems that it is a change in baseline ecological condition that contributes to the degradation of reef community. The literature is replete with the effects of gross alteration of these parameters on numerous individual species and the subsequent effect on the reef communities.

It can be theorized that "healthy" reefs will develop given optimal, baseline, environmental conditions while there will be predictable decay for reefs subject to poor environmental conditions or after acute changes. While the detrimental effects of acute storm events on coral communities are usually severe, they are easily evaluated (Woodley et al., 1981, Done et al., 1986, Goreau and MacFarlane, 1990, Fitt et al., 1993, Lirman and Fong, 1995). However, the effects of subtle, chronic changes in environmental condition on coral communities are more difficult to assess.

The key to identifying community level change resultant from chronic environmental stress may lie in the examination of individual level response to sub-lethal stress over time. To address the role of chronic stress in coral community change, Peters and Pilson (1985) suggest an integrated approach of repeated sampling of random individuals. Peters and Pilson (1985) further suggest that this sampling should include visual assessment of stress response, physiological evaluation of the stress response and histological examination of corals subject to the stress. A brief review of previous research on sedimentation stress provides a description of the current state of coral, stress-response research and may provide insights regarding potential areas of future study.

The Effects of Sedimentation on Hard Corals

The role of sedimentation, a common natural and anthropogenic environmental stress, on coral reef ecosystems and organisms has been studied since early in this century. The pioneering work of the Great Barrier Reef Expedition (Yonge, 1930, Yonge and Nichols, 1931) and other early investigations (Vaughan, 1915, Mayer, 1918, Marshal and Orr, 1931) laid the framework for all future study of sedimentation effects on hard coral and associated reef communities.

These early experiments explored the effects of sediment loading on reef coral through the direct application of sediment to coral colonies both in the field (Mayer, 1918, Edmonson, 1929, Marshall and Orr, 1931) and in the laboratory (Edmondson,

1929). Vaughan (1915) experimented with manipulated environmental conditions by transplanting colonies into different habitats. The evaluation of sedimentation effects on corals in these early investigations was typically presented as a measure of coral colony mortality (Brown and Howard, 1985).

Since that time, numerous experiments, observations and monitoring projects have identified the common effects of sedimentation on reef coral communities. Rogers (1990) describes these effects at the community level as: "decreased species diversity, less live coral [cover of reef area], lower coral growth rates, greater abundance of branching forms, reduced coral recruitment, decreased calcification, decreased net productivity of corals and slower rates of reef accretion."

At the individual colony level the effects of sedimentation range from total recovery (Edmondson, 1929, Thompson et al., 1980, Rogers, 1983, Rice and Hunter, 1992) to death (Mayer, 1918, Marshall and Orr, 1931, Rogers, 1983). Sub-lethal effects of sedimentation at the organism level have also been shown to include: decreased Photosynthesis:Respiration (P:R) ratio (Dallmeyer et al., 1982, Abdel-Salam and Porter, 1988, Telesnicki and Goldberg, 1995); decreased calcification (Szmant-Froelich et al., 1983); decreased growth rate (Dodge, et al., 1974, Dodge and Vaisyns, 1977); increased incidence of bleaching (Bak, 1978, Dallmeyer et al., 1982); increased mucus production (Yonge and Nichols, 1931, Bak, 1978, Rogers, 1979) and decreased reproductive success (Johannes, 1975, Dodge and Vaisyns, 1977, Bak, 1978, Rogers, 1990).

Brown and Howard (1985) and Rogers (1990) have summarized the stressful effects of sedimentation on corals and they address the tremendous differences in coral response to sediment stress. Their reviews provide numerous cases of resistance to, and recovery from, severe sedimentation in both the field and laboratory. While, in other instances, small changes in sediment regime have resulted in the destruction of some previously healthy reefs (Loya, 1976, Hands, et al., 1993, van Katwijk, et al., 1993).

This "paradox" of effects may result from a failure to adequately reflect matters of temporal scale when considering changes in reef community structure (Harriott, 1994). Traditional, visual, benthic sampling of coral community structure provides suitable data on longer time scales, but not the physiological and histological data which Peters and Pilson (1985) suggest is necessary to link individual coral response to community change. One limitation of bridging the gap of scale has been a lack of a suitable, minimally destructive index of distress which can evaluate coral condition over short time scales which is also relevant to long-term changes in condition at the individual and community level.

Evaluating Sedimentation Effects in Hard Coral

The scientific community continues to expand upon early investigations with efforts to quantify and describe the effects of sedimentation stress on hard coral. Previous summaries of the effects of sedimentation on corals and coral reef ecosystems have been done by Johannes (1975), Dodge and Vaisyns (1977), Bak (1978), Brown and Howard

(1985) and Rogers (1990). In addition to a survey of stress effects, Brown and Howard (1985) describe common research methodologies and experiments. Previous techniques to evaluate the role and effect of sedimentation stress on hard coral can best be described as following one of four basic patterns: (1) Direct application of sediment to coral colonies to assess colony-level impacts; (2) Shading and turbidity experiments to evaluate colony and reef-level impacts to increased sedimentation conditions; (3) Correlation studies to assess the colony and reef-level impacts change under various ecological conditions; and (4) Evaluations of colony and reef-level change after catastrophic sedimentation events.

Direct application experiments

Investigations designed to examine individual colony response to sediment loading typically employed the method of direct application of sediments to experimental coral colonies (Table 1). These experiments were conducted in the field and in the laboratory. Sediments were collected and applied to the coral in variable quantities according to the study design and were assumed to represent natural and anthropogenic sedimentation. Treatment colonies were examined and evaluated for changes in condition after application of the sediment usually over short time scales (hours, days and weeks). Observation of change in experimental colonies were measured in various ways including, percent mortality, percent tissue bleaching, change in growth rate (calcification), sediment rejection efficiency, and change in the P:R ratio.

Table 1. Representative summary of previous studies that examined individual colony response to sediment loading through direct application of sediments to experimental coral colonies.

Sediment Application	Region or Species	Evaluation	Results	Reference
Direct	Australia Great Barrier Reef	Mortality/Observation	Range: no affect to death	Mayer, 1918
			Range: no affect to death	Edmundson,1929
			Tolerant to low dose, but die under heavy dose/burial	Marshall and Orr,1931;
			Described sediment removal	Yonge, 1931;
Direct	Caribbean	Sediment rejection	Rejection effeciency determined by size/weight of sediment	Bak and Elgershuizen, 1976
Direct	Australian Great Barrier Reef	Sediment rejection	Range: no affect to death Rejection efficiency not correlated with sediment tolerance	Stafford-Smith and Ormond, 1992 Stafford-Smith, 1993
Direct	Caribbean	Mortality, observation	Measured/compared sedimentation rates Partial tissue death: *A. palmata, M annularis*	Rogers, 1983
Direct burial and suspended sediments	Florida Patch Reefs	LT50 survivial, growth	No long-term effects of suspended sediment. Increased LT50 values after 7 days of sediment burial	Rice and Hunter, 1992
Suspended sediments	Florida Keys	Respiration: Photosynthesis	P:R ratio falls below 1.0 in *A. palmata, Diploria strigosa, M. annularis*	Abdel-Salam et al., 1988

Turbidity experiments

Effects of turbidity on hard corals have been evaluated in the field by shading a coral reef community (Rogers, 1979) Or directly measured in the laboratory by placing experimental colonies in aquaria which contained different levels of turbid seawater (Telesnicki and Goldberg, 1995).

The shading method (after Rogers, 1979) simulates high turbidity conditions by placing opaque material over the treatment colonies. Care is taken to position the frames of the shading structure to minimize changes in flow regime. Level of shading is measured using a light meter and the shading is assumed to imitate conditions of extreme turbidity. Observation of colony and reef-level effects are measured on time scales of weeks to months and can include; percent mortality, percent tissue bleaching and changes in community structure.

Telesnicki and Goldberg (1995) measured coral response to measured levels of turbidity by placing corals in experimental chambers which contained water at three (3) different levels of increasing turbidity (measured as nephelometric turbidity units NTU). Additionally, a control group was placed in an identical experimental chamber containing 0.2 NTU water as a control group. The levels of turbidity corresponded to common levels of turbidity found in the coastal waters of south Florida. Observations were made in relatively short time scales (hours to days) and included the measurement of P:R ratio.

hese two experiments, and the suspended sediment studies by Dallmeyer et al. (1982) and Abdel-Salam et al. (1988) are representative of the few published studies designed to evaluate the role of decreased light levels on hard coral.

Correlation studies

Numerous studies (Table 2) have described changes in coral reef community structure, and the implied or observed effects of sedimentation, relative to changes in environmental condition. These studies correlated long-term environmental change in sedimentation regimes and are typically evaluated over relatively long time scales (years and decades). The most common evaluation method for these studies was the observation and comparison of established transects (including some, which have become extensive, providing continuous data over many years). Long-term assessment of species diversity and percent live cover for each set of transects were correlated with environmental parameters that are monitored independently of the reef communities. Current use of advanced video technology, remote sensing and Geographic Information System (GIS) databases have aided in the ease and usefulness of this data in coral reef management.

Long-term data sets are important in measuring significant trends and changes in reef community structure subject to changing environmental stress, yet relevant information is obtained only after large numbers of the coral have died. The long-term nature of these studies, therefore provide useful, historical data but do not provide insight for resource management issues which need to be addressed on short time scales (regulation implementation or removal).

Table 2. Representative summary of previous studies that evaluated coral community response to sedimentation through correlation of physical conditions and coral community "health."

Location	Situation	Physical Parameters	Evaluation Criteria	Results	Reference
Caribbean	Resuspended sediments	Sedimentation rate	Growth (x-radiography)	Decreased growth rate with increased resuspended sediments	Dodge et al., 1974
Caribbean	Sedimentation and turbidity	FTU, Light, Current, Water Temp., Sedimenation rates	Community structure (% live cover, species diversity)	Increased number of efficient sediment rejection species with increased turbidity and TSS	Loya, 1976
Caribbean	Dredge sediments	Historical dredge data	X-radiography and relative size and abundance of colonies	Dredging/turbidity has long-term detrimental effects on reef coral community structure	Dodge and Vaisyns, 1977
Pacific (Hawaii)	Sewerage diversion	Nutrient levels, chlorophyll levels,	% live cover, species diversity	Decreased species diversity	Smith et al., 1981
Caribbean (Costa Rica)	Sedimentation and turbidity	Water temp, suspended solids, sedimentation rates	Growth rate, larval recruitment	Growth rate inversly correlated with level of resuspended sediments	Cortes and Risk, 1985
Caribbean	Turbidity and sedimentation	Light, sedimentation rate	Growth rate	Lower growth rate in *M.annularis* subject to increased turbidity and TSS	Hubbard and Scatauro, 1985
Caribbean	Environmental factors	Change in Diadema, sedimentation	% live cover, species diversity	Greater prevailance of *M.annularis* in turbid zones	Liddell and Ohlhorst, 1987
Florida Keys	Environmental Factors		% live cover, species diversity	Loss of coral abundance and diversity with increased stress	Porter and Meier, 1992
Costa Rica	Sedimentation	Sedimentation rate	% live cover, species diversity	Decreased live cover, increased prevailance of *D. strigosa, M. annularis* and *Siderastrea radians* with increased sedimentation	Hands, et al., 1993
Indian Ocean (Kenya)	Sedimentation (River discharge)	Sediment fraction	% live cover, species diversity	Correlation between increased coral stress responses and level of terrigenous sedimentation	van Katwijk, et al., 1993

Table 3. Representative study of previous studies that evaluated coral response to sedimentation and turbidity through pre/post comparisons of coral related to catastrophic events.

Event	Location	Evaluation parameters	Results	Reference
Hurricane	Jamaica	Mortality, observed damage	Detachment, abrasion, burial. Massive heads less susceptible to damage.	Woodley et al., 1981
Cyclone	Pacific	Ecological and geomorphological effects	Detachment, abrasion, burial.	Done et al., 1985
Bleaching Event	Jamaica	Growth rate	Reduced growth rate during bleaching event	Goreau and MacFarlane, 1990
Bleaching Event	Florida Keys	Chlorophyll, zooxanthellae densities, protein and lipid	Reduced tissue biomass, protein and total lipid after recovery	Fitt et al., 1993
Hurricane	Florida Keys	Mortality, observed damage	Storm damage from successive storms may be cumulative.	Lirman and Fong, 1995

Catastrophic event analysis

The effects of natural and anthropogenic catastrophic events on coral colonies and communities are critical, non-experimental assessments of sedimentation effects on coral (Table 3). The effects are usually evaluated through observation of damage and degradation at the individual and community levels. These evaluations can be explored over relatively short (days to weeks) to relatively long (months to years) time scales. Pre- and post-event data of percent live cover and species diversity are observed and compared for permanent transects. Post-event observation of colony damage has also aided in determining the deleterious effects of severe sedimentation on coral and coral communities.

Bleaching: A natural coral response to environmental stress

Within the past decade, mass coral bleaching events have captured the attention of scientists, resource managers and other environmental groups. While extreme starvation (Yonge and Nichols, 1931) and sedimentation (Rogers, 1983, 1990) have been shown to induce bleaching, it more commonly results from small changes in water temperature above mean summer levels (Glynn and D'Croz, 1990, Jokiel and Coles, 1990, Fitt et al., 1993, Fitt and Warner, 1995, Lesser, 1997). Bleaching can also result from exposure to high levels of ultraviolet radiation (Lesser et al., 1990, Kinzie, 1993) or the synergistic association of these two factors (Jaap, 1979, Fisk and Done, 1985, Cook et al., 1990, Glynn, 1991, Fitt et al., 1993).

Hard coral possess a variety of heat-stress responses (Fitt and Warner, 1995). As elevated temperatures persist beyond short time scales coral responses range from reducing photosynthetic pigments within individual zooxanthellae (Hoegh-Guldberg and Smith, 1989, Fitt and Warner, 1995) to the complete expulsion of algal symbionts (Jaap, 1979, Lesser, 1997). Mechanisms controlling these responses are not well understood (Fang et al., 1997) but recent studies indicate that bleaching may be triggered by oxidative stress due to overproduction by the zooxanthellae (Lesser et al., 1990, Lesser, 1997) initiated by a disruption of the dark phase of photosynthesis (Jones et al., 1998). Regardless of the mechanisms which trigger bleaching, sub-lethal effects of the condition include a net decrease in photosynthetic potential (Fitt and Warner, 1995) and a reduction in the photosynthesis to respiration (P:R) ratio; with abnormally high respiration (> 9 days) after temperatures return to normal (Hoegh-Guldberg and Smith, 1989, Fitt and Warner, 1993). Longer-term effects of bleaching include decreased growth rate (Goreau and MacFarlane, 1990, Leder et al., 1991), a decreased ability to regenerate damaged tissue (Meesters and Bak, 1993) and decreased reproductive potential and success (Szmant and Gassman, 1990). These effects continue for several months after the corals begin to restore zooxanthellae to normal levels and indicate a correlation between the presence of an adequate number of zooxanthellae and coral growth, tissue maintenance and reproduction.

Under non-stressed conditions, the symbiotic relationship enables host tissue to accumulate large quantities of storage lipid through translocation of fixed carbon from photosynthesis (Patton et al., 1977, Harland et al., 1993). The zooxanthellae translocate

up to 90% of daily fixed carbon to the host tissue (Crossland et al., 1980, Muscatine et al., 1984) which appears to constitute overproduction of lipid (Stimpson, 1987). This rapid production, translocation and storage of fixed carbon as lipid may be necessary due to the rapid use and loss of lipid by coral tissues (Stimpson, 1987). In addition to requirements of normal metabolism and the production of lipid rich eggs and planulae in reproduction, corals lose up to 50% of excess lipid in mucus daily (Crossland et al., 1980, Muscatine et al., 1984, Battey et al., 1998). Patton et al. (1977) suggest that the storage of lipids may enable coral to survive stress events with minimal disruption of the symbiotic relationship, yet turnover of lipid is rapid (Patton et al. 1977, Crossland et al., 1980, Muscatine et al., 1984, Battey et al., 1998).). The lost, or decreased ability to gain fixed carbon from zooxanthellae may result in the decreased growth, tissue maintenance and reproductive abilities associated with bleaching, and may be evident as a function of nutritional condition and the amounts of storage lipids available for use during the stressful event.

Coral Tissue Lipid as an Indicator of Coral Response to Environmental Stress

Total lipid and lipid subclass

Lipid is the primary energy storage compound in hard coral and has been shown to represent a significant proportion of coral tissue mass. Previous efforts to identify a minimally destructive index of coral condition have focused on the evaluation of lipid content (Stimpson, 1987, Harland et al., 1993, Harriott, 1994). Lipids are important in

maintaining the coral symbiosis (Stimpson, 1987), and therefore are associated with growth, tissue repair and reproduction (Ducklow and Mitchell, 1979). The accurate measurement of total lipid is difficult due to large amounts of error associated with achieving accurate tissue weights and is considered too highly variable as a valid indicator of condition (Harriott, 1994).

The analysis of lipid subclass ratio has been used as an indicator of condition in both vertebrates (Pollero et al., 1991, Håkanson, 1993) and invertebrates (Emmett et al., 1987, Fraser, 1989) and may provide the suitable index of coral stress. Lipid ratio measures the relative amounts of storage lipids (subject to metabolic use) and structural lipids (relatively stable over short time scales) and provides a dimensionless value that is not subject to the large sampling error associated with quantifying total lipid based upon measurements of tissue sample weight.

Corals, like other organisms in nutrient limited environments, must balance the nutritional expense of reproduction against the nutritional demands of growth, development and normal metabolism (Ward, 1995). Excess nutrient expense or nutrient deficiencies can deplete nutrient reserves and depress, or eliminate, reproductive activity, growth or development. Nutrient reserves are stored as wax esters (WE) and triacylglycerides (TAG). Levels of WE and TAG can fluctuate with coral reproductive state (Stimpson, 1987), food availability, metabolic requirements (Crossland et al., 1980, Crossland, 1987) and productivity of algal symbionts (Patton et al., 1977, Stimpson, 1987). The sterol esters (SE) and the polar lipids (including the phospholipids, PL) are

structural lipids and should remain stable with short-term changes in nutritional state. The relative amounts of storage lipid to structural lipid may provide insight to the nutritional condition of coral colonies under short time scales which have ramifications over longer time scales (decreased reproduction, decreased growth, decreased calcification).

Qualitative measurements of coral tissue lipids are easily recovered using a simple methanol and chloroform extraction modified after Folch et al. (1957) and Bligh and Dyer (1959). Scleractinians have been shown to be rich in tissue lipids with the majority lipid present being WE or TAG (Harland et al., 1993). *Montastrea annularis* has been shown to contain 32% of its dry weight as lipid, with wax ester constituting 42% of dry weight and 65% total lipid (Harland, et al., 1993). These ratios fluctuate under environmental condition and among species (Meyers et al., 1978a, 1978b, Latyshev et al., 1991, Harland et al., 1993).

Fatty acid analysis

The relative contribution of zooxanthellae-fixed carbon and fixed carbon from hetertrophic feeding has been analyzed for *Montastrea annularis* and other species of Caribbean reef coral under steady-state environmental conditions (Meyers et al, 1978a, 1978b). Fatty acid found in the tissues of hermatypic coral relflect lipid components from both the host and the zooxanthellae. Although the relative contribution of zooxanthellae to the nutritional budget of the host has been difficult to quantify, changes in these levels under stressed conditions may provide further insight into the pathways affecting nutritional state and degradation of that state.

Goals of this dissertation

I will explore the use of lipid subclass ratio as an indicator of environmental stress in the scleractinian corals *Montastrea annularis* and *M. faveolata* on short time scales. In addition to developing techniques to measure of coral condition I hope to shed insight to the pathways and mechanisms of the nutritional expense of environmental stress through the use of fatty acid analysis. I will also attempt to refine this work to broaden the use of tissue lipid subclass analysis as a monitoring tool for hard corals through the development and testing of techniques to limit the destruction of large amounts of coral skeleton and tissue. Minimal destruction of coral tissue will permit repeated sampling of the same individual colonies over time. Repeated sampling is an essential element of any coral reef monitoring program and may provide a link between individual short-term response and long-term community change.

Choice of experimental organism

Montastrea annularis and its related species *M. faveolata* are primary frame-building corals of the Caribbean region (Goreau, 1959) and are therefore some of the most important scleractinians in Caribbean reef communities. In addition to the level of importance based entirely their role as a reef-builders, *Montastrea spp. are* often subject to destructive natural and anthropogenic sedimentation stress due to colony morphology and their relatively shallow growth range in the water column. These features, in addition to previous work which has analyzed lipid subclass levels (Harland, et al., 1993) and fatty acid analysis (Meyers, et al., 1978a, 1978b.) in *M. annularis* under non-sediment stress conditions make this an excellent research organism for this work.

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CHAPTER II

ENVIRONMENTAL STRESS AND HARD CORALS: RE-EVALUATING LIPID AS AN INDICATOR OF SEDIMENT STRESS IN *MONTASTREA ANNULARIS* AND *MONTASTREA FAVEOLATA*

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ABSTRACT

Lipid quality was evaluated in *Montastrea spp.* under sediment-stressed conditions. Levels of total lipid in *M. annularis* subject to experimental sedimentation were highly variable (10.07 - 210.37 mg lipid/g dry tissue) and did not differ significantly with treatment ($p > 0.25$). The ratio of storage lipid (wax ester + triacylglyceride) to structural lipid (sterol esters + phospholipid) decreased significantly (from 0.25 to 0.14, $p < 0.01$) in treatment colonies and compared to control levels. Fatty Acid (FAME) analysis of colonies of *M. annularis* exposed to experimental sedimentation showed a reduction of the algal, 18:3(n-6) and 18:4(n-3), polyunsaturated fatty acid (PUFA) in the polar fraction of tissue lipid extracts. This loss of PUFA suggests a loss of algal membrane in sediment-stressed colonies. Lipid quality was similarly measured in 20 colonies of *M. faveolata* over a 10-day period. Mean (n=20) ratio of storage to structural lipid in *M. faveolata* dropped from 2.43 to a level of 0.98 immediately following a natural sedimentation event before recovering to levels of 1.4 and 2.9 post-storm days 2 and 4, respectively ($p < 0.01$). Under conditions of sedimentation stress *Montastrea annularis* and *M. faveolata* both exhibited a significant, immediate decrease in the level of storage to structural lipids. The lipid ratio may provide a useful tool to monitor and assess individual coral condition during periods of sub-lethal environmental stress.

INTRODUCTION

Sedimentation effects on hard coral have been evaluated since early in this century (Vaughan, 1915, Mayer, 1918, Marshall and Orr, 1931) and have been summarized by Johannes (1975), Dodge and Vaisyns (1977), Bak (1978), Brown and Howard (1985) and Rogers (1990). At the individual colony level, the effects of sedimentation range from total recovery (Edmondson, 1929, Thompson and Bright, 1980, Rogers, 1983, Rice and Hunter, 1992) to death (Mayer, 1918, Marshall and Orr, 1931, Rogers, 1983). Sub-lethal effects of sedimentation at the organism level have also been shown to include decreased photosynthesis to respiration (P:R) ratio (Dallmeyer et al., 1982, Abdel-Salam et al., 1988, Telesnicki and Goldberg, 1995); decreased calcification (Szmant-Froelich et al., 1981); decreased growth rate (Dodge et al., 1974, Dodge and Vaisyns, 1977, Tomascik and Sander, 1985); increased incidence of bleaching (Bak, 1978, Dallmeyer et al., 1982); increased mucus production (Yonge, 1931, Bak, 1978, Rogers, 1979) and decreased reproductive success (Johannes, 1975, Dodge and Vaisyns, 1977, Bak, 1978, Rogers, 1990). Rogers (1990) describes the effects of sedimentation at the community level as: "decreased species diversity, less live coral [cover of reef area], lower coral growth rates, greater abundance of branching forms, reduced coral recruitment, decreased calcification, decreased net productivity of corals and slower rates of reef accretion." While individual and community responses have been studied separately, there has been little success in determining the relationship between individual-level response, and community-level response to sedimentation stress (Harriott, 1994).

Peters and Pilson (1985) address this problem and suggest a list of parameters for evaluating coral community response to environmental stress through the long-term monitoring of random individuals. The list includes 1. Visual observations of individual colonies (polyp condition, lesions, mucus, bleaching, presence of sediments); 2. Histological examinations (reproductive cycle and development, morphological changes, presence of microparasites or pathogens) and; 3. Physiological measurements (metabolic rate, photosynthesis rate, calcification and growth rates, biochemical composition). While long-term visual observations and monitoring have continued to provide valuable information there has been relatively little biochemical and other cellular-level research designed to quantify sub-lethal responses to environmental stress.

However, recent studies have begun to address the issue of biochemical and cellular level change. P:R ratio has been shown to decrease in corals under sediment stress (Abdel-Salam et al., 1988) and increased turbidity (Telesnicki and Goldberg, 1996). Corals subject to thermal stress have been shown to produce heat-shock protein and increased levels of intracellular calcium (Hayes and King, 1995, Fang et al., 1997). Many authors have suggested evaluation of coral tissue lipid as an indicator of coral condition for a variety of environmental stresses (Stimpson, 1987, Harland et al., 1993, and Harriott, 1994).

Lipids constitute the bulk of the energy storage compounds in coral (Ducklow and Mitchell, 1979) and are linked to essential functions of growth, development, sexual

reproduction and tissue repair. Like other organisms in nutrient limited environments, corals must balance the nutritional expense of reproduction against the nutritional demands of normal metabolism (Ward, 1995). Excess use of energy reserves or nutritional deficiencies can deplete nutrient reserves and depress, or eliminate, reproductive activity, growth and development. These nutrient deficiencies are manifest on the organism level during the short-term (bleaching, limited sexual reproduction, limited ability to repair tissue, low growth rate) but may also be evident in the community level, over long-time scales as changes in species diversity and percent live cover (Tomascik and Sander, 1987, Ward, 1995).

Reef-building corals store nutrient reserves as wax esters (WE) and triacylglycerides (TAG)(Patton et al., 1977, Harland et al., 1992). Levels of WE and TAG can fluctuate with coral reproductive state (Stimpson, 1987), productivity of algal symbionts (Patton et al., 1977, Stimpson, 1987), metabolic requirements and food availability (Crossland et al., 1980, Crossland, 1987). The sterol esters (SE, primarily cholesterol) and the polar lipids (including the phospholipids, PL) are structural lipids and remain relatively stable with short-term changes in nutritional state (Meyers, 1979). Changes in lipid class abundance will be manifest in the storage lipid (through use or decreased production of photosynthate) while the levels of structural lipids should remain constant on short time scales. These fluctuations in lipid class levels provide the basis for evaluation of lipid ratio as an indicator of coral condition given the previously reported coral responses to sedimentation stress.

The use of total lipid as an indicator of coral condition has been criticized as unreliable and highly variable (Harland et al., 1993, and Harriott, 1994). The relative amounts of storage lipid to structural lipid (WE+TAG):(SE+PL) has been used as a condition index in other organisms. Pollero et al. (1991) and Fraser (1983) describe the relationship between adequate storage lipid and successful development in fish, bivalve and crustacean larvae and Emmett et al. (1987) correlate levels of TAG and nutritional condition in mussels. Håkanson (1993) studied lipid ratio larval anchovies and was able to compare the nutritional condition of the larvae with environment. Lipid ratio may therefore provide insight into the nutritional condition of individual coral colonies under short time scales. The use of a lipid ratio provides a dimensionless index of lipid quality. The comparison of storage lipids (energy) to structural (membrane) lipids provides information about the nutritional state of the coral (i.e. the presence of adequate energy stores for reproduction) without first determining the quantity of lipid in the sample and its use eliminates dependence upon tissue sample weight. Conversely, total lipid is difficult to accurately measure in hard corals for many reasons including small, difficult to obtain tissue samples, remnant calcium carbonate skeleton which affects sample weight and loss of lipid in sample preparation (Harriott, 1994). Total lipid studies require the amount of lipid to be expressed as lipid per unit weight or lipid per unit area, a potential source of large sampling error (Harriott, 1994), whereas lipid ratio is based solely on the relative quantities of lipid subclass for a given sample.

Analysis of the lipid sub-units, fatty acids, may provide insight into the relative contribution of zooxanthellae and host tissue in response to sub-lethal stress. Fatty acids consist of long carbon chains that maintain their structural integrity through trophic levels. This structural integrity allows fatty acids to be used as biomarkers in feeding studies. For example, only plants and phytoplankton can produce linolenic acid (18:3n-3) and linoleic acid (18:2n-6) and large amounts of PUFA are indicative of marine plants and dinoflagellates (Meyers et al, 1978, Meyers, 1979, Harland et al., 1993). Little data has been collected on coral fatty acids and the relative abundance of saturated fatty acids (no double bonds between carbon atoms), unsaturated fatty acids (one double bond) and polyunsaturated fatty acids (PUFA, $\geq$ 2 double bonds) remains unresolved (Harland et al., 1993). However, the saturated fatty acids, palmitic acid (16:0) and stearic acid (18:0) are the primary fatty acids in coral (Meyers, 1979) and recent research by Al-Moghrabi et al. (1995) indicate the importance of arachidonic acid (20:4 n-6) and docosatrienoic (22:3 n-3) PUFA in corals subject to stress.

The objectives of this study include an examination of lipid response to sedimentation stress over short time scales. This report describes the change in tissue lipid subclass ratio in the hard corals *Montrastrea annularis* and *Montastrea faveolata* after experimental and natural sedimentation, respectively. The lipid subclass ratio was further evaluated for consistency and accuracy to assess the usefulness of this method in evaluating nutritional condition in these species. Analysis of fatty acid composition was completed to investigate the relative changes in algal- and host-derived fatty acids in corals subject to sedimentation stress.

MATERIALS AND METHODS

Experimental sedimentation study

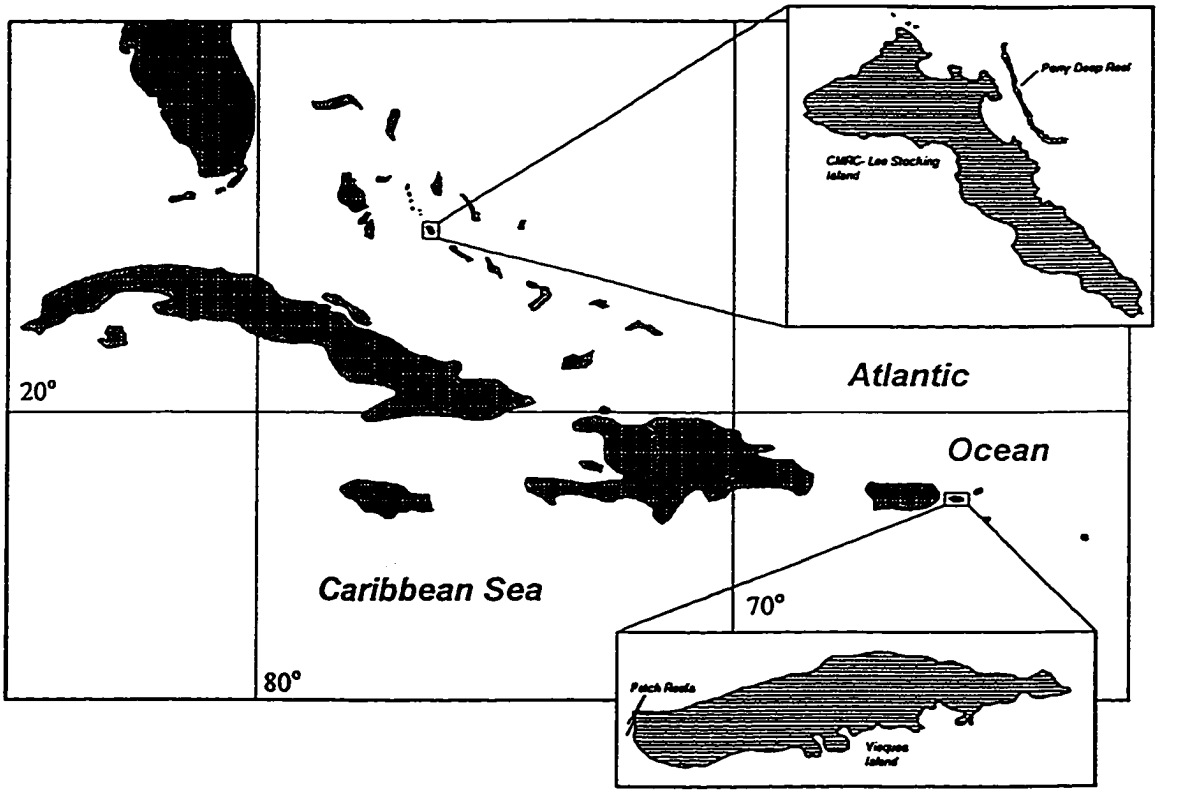
Ten colonies of *Montastrea annularis* were haphazardly selected from small, leeward patch reefs (depth = 3.0 m) adjacent to the northwest coast of Vieques, Puerto Rico (18°06'34" N, 65°34'32"W)(Figure 1) and marked for use (5 control and 5 treatment) in the sedimentation experiments from July 14-19, 1997. Tissue samples were collected by a SCUBA diver using a geologist's pick, by removing a small (3.0–8.0) cm^2 section of tissue and skeleton from each colony. Each section of coral was individually placed in a Whirlpak® collection bag containing seawater and stored in a cooler for return to the laboratory. Locally obtained sediments were collected, dried and weighed to determine an approximate, standard weight/area of 5.0 mg/cm^2 for each dose using a pre-marked scoop. A dose of 5.0 mg/cm^2 was chosen because it approximates the level of sediment loading Rogers (1990) describes as causing sub-lethal effects on hard coral communities. Sediment was applied to the central portion of 5 experimental colonies after Rogers (1983). Five control colonies did not receive any applied sediment. After 24 hours, post-treatment samples were removed, as above, from the same area on each coral lobe to countermand possible clade effects. Upon return to the laboratory each sample was individually preserved in a solution of 10% Formalin and filtered seawater (1/10 v/v) for 24 hours. After preservation, the sample was rinsed in filtered seawater and frozen, at –20°C, for transfer, on ice, to the Virginia Institute of Marine Science. Samples were stored at –20°C until processing.

Five replicates of coral tissue (3-5 adjacent polyps) were removed from the carbonate skeleton of each of the colonies with fine forceps, placed in pre-weighed amber collection vials, and freeze-dried in a VIGRIS (Gardiner, NY) desiccator/freezer. The vials were re-weighed to obtain a dry weight of the tissues and remained frozen until processing.

Lipid Class Analysis

Lipid class content was determined using an Iatroscan thin layer chromatograph (TLC) with a flame ionization detection (FID) system (Iatroscan Laboratories Inc., Tokyo Japan). Coral tissue lipids were extracted twice from homogenized polyp tissues using the modified methods of Folch et al. (1957) and Bligh and Dyer (1959). Briefly, this procedure separates and extracts lipids from tissue in a biphasic solution of chloroform/methanol/water (2:2:1, v/v). Stearyl alcohol was added to the samples prior to extraction as an internal standard to correct for extraction efficiency. Approximately 10-25 mg of dry sample was extracted for lipid class analysis. The lipid phase was captured, pooled for the two extractions, and evaporated to dryness to concentrate the sample to 100 µl. Two (2) µl of concentrated lipid extract were spotted on S-III chromarods and lipid classes were developed in hexane:diethyl ether:water (85:15:0.04) to separate the lipid classes. The chromarods were scanned and lipid classes were quantified by standard curve on using a Mark III Iatroscan analyzer.

Figure 1. Location of study sites at the Perry Reef, Lee Stocking Island, Exuma Cays, Bahamas and at Vieques, Puerto Rico.



Fatty Acid Methyl Esterization (FAME)

Tissue lipids were extracted as described above. Lipids were re-extracted in a solution of DCM:MeOH (98:2). Neutral lipids (wax ester, triacylglyceride, sterol esters) were separated from polar lipids (phospholipid) using a silica gel micro-column and rinsed to 13 ml with DCM:MeOH (98:2) and MeOH respectively. Each fraction was collected in a graduated vial containing 5.0 µl of C 23:0 as an internal standard. Methyl esterization of the fatty acids was accomplished with Boron trifloride (BF_3). The FAMEs were extracted and cleaned with carbon disulphide (CS_2) and water. Samples (5.0 µl) were analyzed using a VARIAN 3300 Gas Chromatograph (Varian Inc., Walnut Creek, CA) coupled with a flame ionization detector. FAMES were identified determined using comparisons to retention time and quantities of internal standards.

Natural Sedimentation Study

Coral lipid response to storm induced sedimentation was evaluated from 9-19 June 1995 at the Perry Reef (23°46'30"N, 76°05'40"W) (Figure 1), a windward fore-reef, at Lee Stocking Island (LSI), Exuma Cays, The Bahamas. All field collections and initial sample preparation were conducted at the Caribbean Marine Research Center (LSI). Reef depths were within the range of 12-15 meters in the study area. Mean sedimentation rate was determined by the use of five sediment traps placed at the reef base (after Butman, 1986). One tissue sample was collected from each of 20 colonies by a SCUBA diver using a geologist's pick, by removing a 3.0–5.0 cm^2 section of tissue and skeleton from haphazardly selected colonies of *Montastrea faveolata* (Ellis and Solander, 1786) on each

sample date. Samples were collected on days 0 (3 days prior to the storm event), 5 (storm +1 day), 7 (storm +3), and 9 (storm +5days) of the study by SCUBA divers. Samples were not collected on days 3 or 4 because of storm conditions. Each section of coral was individually placed in a Whirlpak collection bag containing seawater and stored in a cooler for return to the laboratory. Coral tissue (3-5 adjacent polyps) was removed from the carbonate skeleton of each of the colonies with fine forceps placed immediately in amber vials containing 1.0 ml of methanol:chloroform (2:1 v/v) and frozen until qualitative analysis at the Virginia Institute of Marine Science. Lipids were extracted and analyzed using the methods described above for the sedimentation experiment.

Data analysis

Coral tissue lipid responses to sedimentation were measured and presented as the ratio of storage lipid to structural lipid (unless otherwise noted) and statistical tests were made using arcsine transformed proportions. Statistical comparisons of experimental results are, unless otherwise noted, paired *t*-tests (two-tailed) of coral colonies subject to similar conditions and tested at the same time. When paired tests were impossible or inappropriate (lost or destroyed "pairs") tissue samples were grouped by condition, or day, and analyzed using a standard ANOVA. The use of individual tissue samples, taken from the same colony, as independent samples has been widely used and tested (Meyers et al., 1978, Meyers, 1979, Latyshev et al., 1991, Harland et al., 1992, Kinzie, 1993). To test for possible effects of pseudo-replication, paired two-factor ANOVA, with one factor set as colony replicate and the other factor as treatment was conducted. The colony replication term was not significant ($p >> 0.25$).

RESULTS

Sedimentation

Experimental colonies of *Montastrea annularis* experienced a one-time episode of sediment loading equal to approximately 5.0 mg/(cm^2). Sediments collected at the Puerto Rico reef site were primarily composed of terrigenous (> 50%) sediments and contained 3% gravel, 90 % coarse and medium sands and 6% fine sand and silt. The colonies of *M. faveolata* subject to storm-induced sedimentation were impacted over a two-day period with a mean, natural, maximum sedimentation rate of 0.04 mg/(cm^2 day) for the storm period. Reef sediments at the LSI site were composed of carbonate (>99%) sediments that were 1% gravel, 97% coarse and medium sands and 2% fine sand with no measurable silts or clays. Background sedimentation rates were 0.0 mg/(cm^2 day) for both experimental and natural sedimentation studies. The experimental treatment dose was considerably greater than the natural sedimentation of the storm event and is consistent to the level of 10.0 mg/(cm^2 day) identified as detrimental to Caribbean reef coral communities by Rogers (1990). Two days previous to the start of the natural sedimentation study, a storm event resulted in increased sedimentation to the study area. No apparatus was in place to measure sedimentation for the preliminary event although this event may have affected *M. faveolata* lipid ratio at the start of the testing period.

Table 4. Lipid subclass totals for control and treatment colonies of *Montastrea annularis*. Quantity of lipid is presented as μg lipid/ mg dry tissue.

	SEDIMENT TREATED SAMPLE								CONTROL (NON-SEDIMENT) SAMPLES							
	Pre-treatment				Post-treatment				Pre-treatment				Post-treatment			
	Lipid	s.e.	%	s.e.	Lipid	s.e.	%	s.e.	Lipid	s.e.	%	s.e.	Lipid	s.e.	%	s.e.
WE	2.13	0.35	9.62	0.84	0.88	0.10	5.27	0.65	4.20	1.65	22.20	2.97	2.68	0.53	11.40	1.21
TAG	2.22	0.49	9.42	1.01	1.07	0.15	6.23	0.88	4.73	2.23	15.80	2.22	2.37	0.37	9.42	0.83
Total Storage Lipid	4.35	0.81			1.85	0.26			8.92	3.86			5.05	0.84		
FFA	0.55	0.13	2.30	0.26	0.23	0.06	0.60	0.21	1.13	0.55	1.35	0.53	0.39	0.14	1.00	0.28
SE	2.78	0.42	11.10	0.95	0.24	0.12	12.80	0.85	4.69	2.08	7.79	1.46	2.56	0.40	11.60	0.44
PL	14.25	1.95	67.50	1.75	12.85	0.67	75.70	2.00	26.07	12.60	52.80	3.14	14.10	2.32	66.60	1.24
Total Structural Lipid	16.73	2.35			14.33	1.10			30.77	14.67			16.66	2.71		
Total Lipid	20.95	3.07			17.01	0.81			40.70	19.01			22.00	3.46		
FFA:Total	0.02	0.00			0.01	0.00			0.02	0.00			0.01	0.01		
(WE+TAG):(SE+PL)	0.25	0.03			0.14	0.02			0.32	0.02			0.31	0.03		
(SE+PL):Total	0.79	0.01			0.89	0.02			0.74	0.01			0.76	0.01		

Figure 2. Mean pre- and post-treatment levels of total lipid (mg lipid/g freeze dried tissue weight ± standard error) for control  and treatment  colonies of *Montastrea annularis* subject to experimental sediment stress. Experimental colonies received a treatment dose of <5.0 mg/cm^2 of locally obtained sediments while control colonies received a treatment dose of 0.00 mg/cm.^{2} (ANOVA, $p > 0.20$)

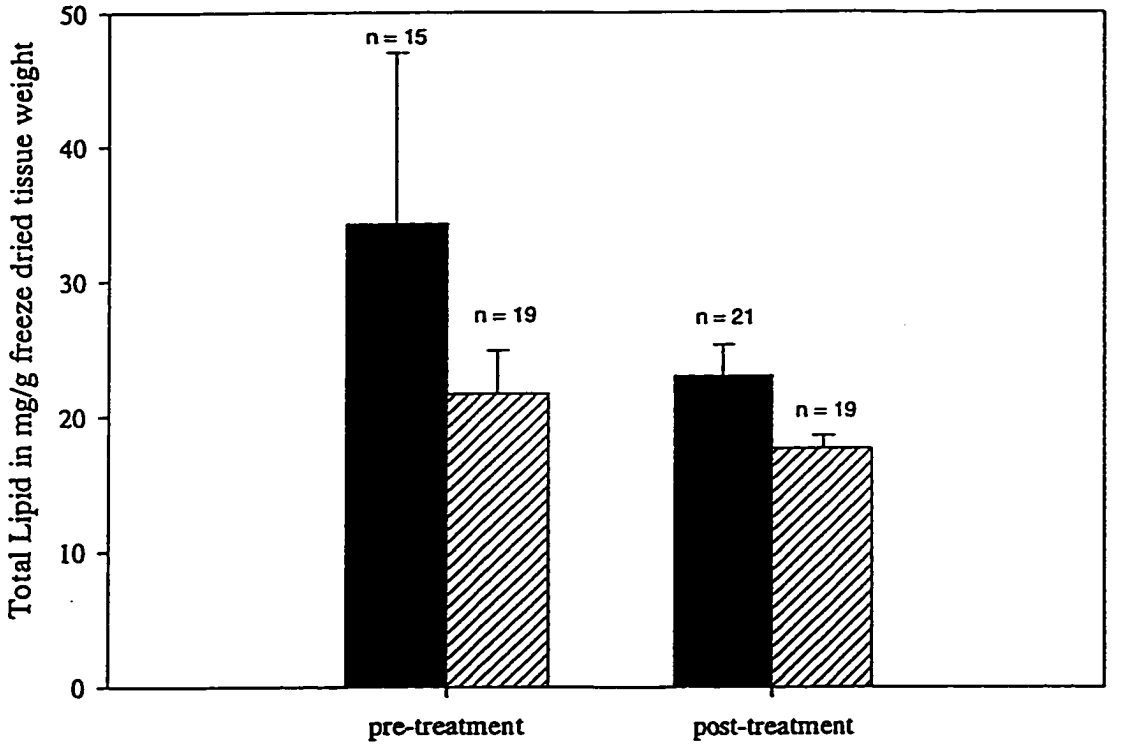


Table 5. Mean Lipid subclass totals for colonies of *Montastrea faveolata* (n = 20) measured for the period 9-19 Jun 1995. Storm and sedimentation event took place on Days 3 & 4. Quantity of lipid is presented as mg lipid/5 polyp sample.

	Pre-Storm (Day 0)				Storm +1 (Day 5)				Storm + 3 (Day 7)				Storm + 5 (Day 9)			
	Lipid	s.e.	%	s.e.	Lipid	s.e.	%	s.e.	Lipid	s.e.	%	s.e.	Lipid	s.e.	%	s.e.
WE	11.27	1.92	48.18	4.32	2.31	0.19	38.40	3.89	1.88	0.4	45.53	3.92	3.42	0.34	72.42	2.00
TAG	1.49	0.45	11.43	4.09	2.01	1.57	7.72	3.67	0.32	0.11	11.32	3.93	0.00	0.00	0.00	0.00
Total Storage	12.76	1.86			4.32	1.58			2.20	0.36			3.42	0.34		
FFA	0.00	0.00	0.01	0.01	2.23	2.17	3.49	2.72	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SE	0.55	0.08	2.85	0.50	1.37	0.8	7.97	3.08	0.12	0.06	3.47	1.59	0.03	0.03	0.38	0.38
PL	6.89	0.73	37.53	4.04	3.14	0.64	42.42	2.88	1.36	0.15	39.68	2.28	1.13	0.04	27.2	2.09
Total Structural	7.44	0.79			4.50	1.43			1.48	0.16			1.16	0.05		
Total Lipid	30.20	2.36			26.05	5.12			3.68	0.50			4.58	0.36		
FFA:Total	0.00	0.00			0.03	0.03			0.00	0.00			0.00	0.00		
(WE+TAG):(SE+PL)	2.43	0.56			0.98	0.11			1.42	0.13			2.92	0.26		
(SE+PL):Total	0.40	0.04			50.40	3.22			43.15	2.29			27.58	2.00		

Figure 3. Mean ratio (± standard error) of lipid subclass of *Montastrea annularis* subject to sediment stress (A) Mean ratio of structural lipid to total lipid (SE+PL:TOTAL LIPID) for pre- and post-treatment condition with no significant differences between pre- and post-treatment ratio (paired, two-tailed *t-test*, $p > 0.25$). (B) Storage lipid to structural lipid (WE+TAG:SE+PL) fell significantly with treatment (paired *t-test*, $p < 0.01$). (C) Free Fatty Acid:Total lipid (FFA:TOTAL LIPID) did not differ between (•) control and (▲)experimental colonies, or with treatment (paired *t*-test, $p > 0.25$).

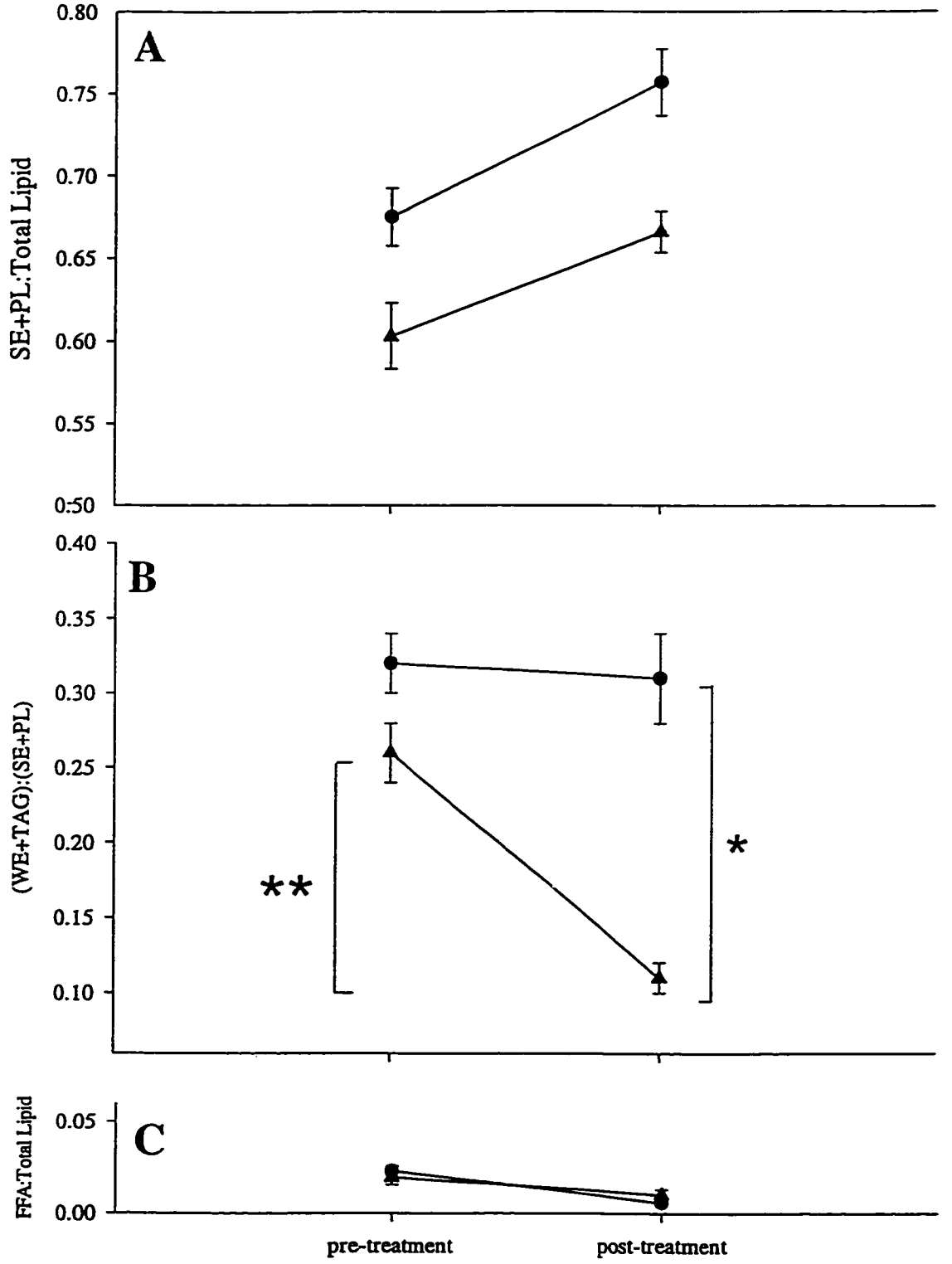
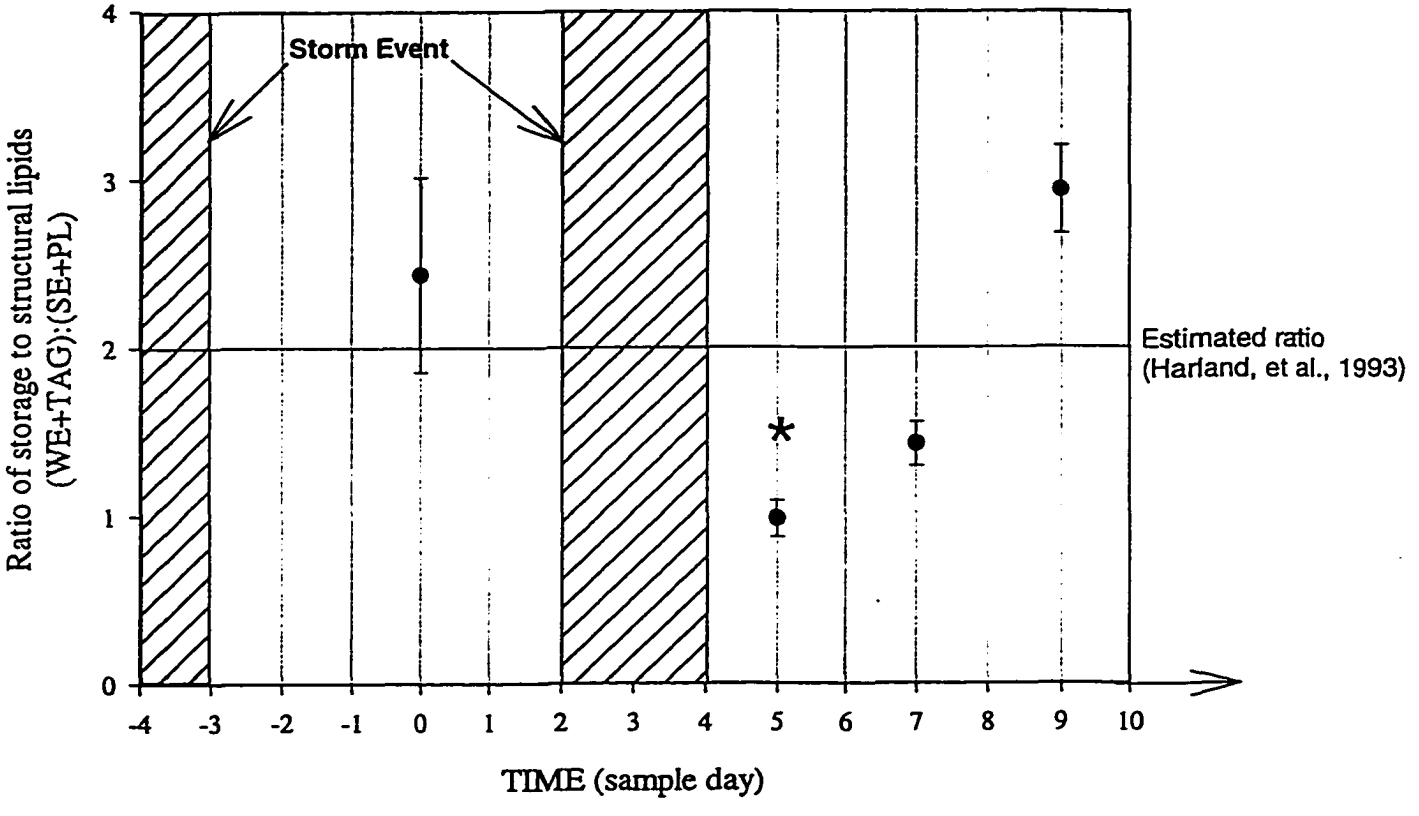


Figure 4. Mean ratio (±standard error) of storage (WE+TAG) to structural (SE+PL) lipid in colonies of *Montastrea faveolata* during the period of June 9-19, 1995. Day 0 represents the start of testing. The storm-sedimentation event occurred on Day 3 and Day 4. Post-storm samples (*) mean lipid ratio dropped significantly from pre-storm and + Day 9 samples (ANOVA $p < 0.01$, Tukey mean comparisons test $p < 0.05$ for Day 5).



Response of M. annularis *to experimental sedimentation*

Total lipid content (mg lipid/g dry tissue weight) varied greatly amony individual coral samples and mean total lipid decreased in both sediment treated and non-treatment colonies. Lipid class composition analysis demonstrated the presence of WE, TAG, small quantities of FFA, SE and PL in *M. annularis* (Table 4). Measures of total lipid were extremely variable (sample range from 10.07 – 210.37 mg lipid/g dry tissue) for each of the treatment colonies despite normalization to freeze dried weight (mg) (Figure 2). Total lipid/dry weight did not vary significantly between pre- and post-treatment corals subject to experimental sedimentation (ANOVA, $p > 0.22$). This variability under both control and sedimentation treatments supports arguments against the use of total lipid as an indicator of sedimentation stress (Harriott, 1993).

Although total lipid was found to be too variable to measure changes in coral nutritional condition, the ratio of (WE+TAG): (SE+PL) in coral colonies exposed to sedimentation (0.14, s.e. = 0.02) differed significantly from pre-test (0.32, s.e. = 0.02) and post-test control colonies (0.31, s.e. = 0.03) and from pre-treatment levels (0.25, s.e. = 0.03) in experimental colonies (paired, two-tailed *t*-test, $p < 0.05$)(Figure 3). Lipid class composition of control and experimental colonies are presented in Table 4. Post-treatments levels of sediment-dosed colonies resulted from decreases in the levels of the storage lipids WE and TAG, while levels of PL and SE remained consistent with pre-treatment levels (Figure 3). The levels of Free Fatty Acids (FFA) were low (ratio of FFA: total lipid: 0.010-0.025, s.e. = 0.003), consistent with previous studies (Patton, et al., 1977, Harland, et al, 1993) and did not fluctuate with treatment (Figure 3).

Response of Montastrea faveolata *to natural sedimentation*

Mean lipid class compositions of *Montastrea faveolata* subject to a natural sedimentation event are given in Table 5. The mean ratio of storage to structural lipids in the initial sampling (Day 0) of *M. faveolata* colonies was 2.43 yet exhibited a large variability (s.e. = 0.58) (Figure 4). The mean ratio (storage: structural) of Day 5 (immediate post-sedimentation) samples decreased significantly to a level of 0.98 (s.e. = 0.11) before returning to 1.4 (s.e. = 0.13) and 2.9 (s.e. = 0.26) on Day 7 and Day 9 respectively (ANOVA $p < 0.01$, Tukey Mean Comparisons Test $p<0.05$ for Day 5).

Fatty Acid Composition of M. annularis

Results of the FAME analysis are found in Table 6. The neutral lipids were predominantly comprised of palmitic acid (16:0) and stearic acid (18:0) (69.2% of the total fatty acids) but contained relatively small amounts of polyunsaturated fatty acid (PUFA). There were no significant differences in neutral lipid fatty acids between control and sediment-stressed tissues. The polar lipid fraction contained the higher portion of PUFA, including the algal-derived 18:3(n-3) and 18:4(n-3) fatty acids. Experimental colonies exhibited a significantly lower level of PUFA 18:3(n-6), 18:3(n-3), 18:4(n-3), 20:5(n-3) and a significantly higher level of the saturated fatty acid 18:0 (two tailed *t*-test, $p < 0.05$) than control colonies.

Table 6. Weight percent fatty acid composition (% total) of the neutral and polar fractions of *Montastrea annularis* subject to two levels of sedimentation stress, Control (0.00 mg/cm^2) and Experimental ($\approx$ 5.0 mg/cm^2). Harland et. al (1993) fatty acid values are given in percentage of total lipid. Significant differences are denoted by (*) (*t*-test, $p < 0.05$). Notation shows the first number of carbon atoms, followed by number of double bonds and then the position of the terminal double bond in relation to the hydrocarbon end of the molecule.

	Neutral Lipids				Polar Lipids				Harland et al. (1993)
	Control		Experimental		Control		Experimental		
		s.d.		s.d.		s.d.		s.d.	
14:0	3.16	0.54	3.62	0.14	1.43	0.68	1.95	0.38	1.10
16:0	59.24	1.62	60.38	2.80	15.04	2.77	18.68	3.11	58.60
17:0	0.15	0.09	0.19	0.09	0.29	0.19	0.45	0.21	
18:0	9.98	0.96	10.34	1.29	9.16	1.82	14.58 *	1.19	12.40
19:0									3.30
16:1(n-7)	2.10	0.19	2.05	0.17	0.87	0.13	0.87	0.13	1.90
18:1(n-9)	5.42	0.88	5.42	0.65	1.73	0.39	2.06	0.29	7.10
18:1(n-7)	1.19	0.67	1.35	0.44	0.99	0.23	1.71	0.17	1.40
20:1(n-11)	0.00	0.00	0.00	0.00	0.00	0.00	0.71	1.00	
20:1(n-9)	1.16	0.18	1.22	0.08	0.91	0.54	0.58	0.06	1.90
20:1(n-7)	0.00	0.00	0.14	0.15	0.00	0.00	0.54	0.77	0.20
16:2(n-7)	0.37	0.10	0.26	0.16	0.60	0.54	0.58	0.31	
16:2(n-4)	0.12	0.21	0.18	0.18	0.98	0.54	0.32	0.04	
16:3(n-4)	0.00	0.00	0.14	0.09	0.27	0.28	0.25	0.19	
16:4(n-3)	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.13	
18:2(n-6)	1.30	0.15	1.15	0.03	1.49	0.42	1.18	0.34	1.20
18:3(n-6)	2.25	0.17	2.17	0.24	8.68	1.06	5.60 *	0.94	
18:3(n-3)	0.00	0.00	0.00	0.00	2.82	0.99	0.64 *	0.72	
18:4(n-3)	0.42	0.09	0.32	0.29	12.06	1.81	6.86 *	0.44	0.20
18:5(n-3)	0.25	0.15	0.17	0.17	7.81	1.11	4.48	0.49	
20:2(n-6)	0.42	0.20	0.39	0.23	0.81	0.74	0.62	0.20	0.30
20:3(n-6)	0.84	0.09	0.61	0.20	0.23	0.23	0.58	0.10	1.40
20:4(n-6)	2.42	0.29	2.29	0.40	12.01	0.76	15.13	0.39	2.20
20:5(n-3)	0.97	0.15	0.54	0.12	3.88	1.06	2.46 *	0.38	0.50
21:5(n-3)	0.02	0.04	0.06	0.11	0.80	0.12	0.51	0.36	
22:4(n-6)	0.81	0.20	1.09	0.29	3.21	0.21	3.03	0.66	
22:5(n-6)	0.00	0.00	0.22	0.22	0.00	0.00	0.00	0.00	0.90
22:5(n-3)	0.26	0.07	0.24	0.10	0.72	0.17	0.58	0.05	0.30
22:6(n-3)	4.88	0.24	3.71	0.72	6.66	0.21	5.87	0.21	2.90
TO.SAT.	73.42	1.04	75.40	1.90	26.19	4.97	36.40	3.10	76.20
TO.MONO	10.10	1.56	10.51	1.29	4.65	0.92	6.68	1.42	13.00
TO.POLY	15.35	0.47	13.60	2.09	63.11	4.61	48.97	0.92	9.90
(n-3)/(n-6)	0.82	0.07	0.61	0.09	0.99	0.07	0.64	0.05	
22:6/20:5	5.17	0.99	7.10	1.99	1.87	0.56	2.45	0.40	
22:5/20:4	0.00	0.00	0.12	0.12	0.00	0.00	0.00	0.00	
TOTAL	97.74	0.00	98.26	0.00	93.45	0.00	90.98	0.00	99.10

DISCUSSION

Lipid ratio

Results of this study clearly indicate that the ratio of (WE+TAG):(SE+PL) decreased in both *Montastrea annularis* and *M. faveolata* after low-level sedimentation. The decrease in the ratio of storage lipids to structural lipids fell consistently under both natural and experimental, sedimentation-stress conditions. Although lipid ratio reflected coral response to sedimentation stress, these results support previous studies that suggested that *total* lipid is a poor indicator of coral nutritional condition due to the large variability of the results.

Stimpson (1987) found that several long-term changes in coral condition could be correlated between environmental conditions, natural changes in coral life-cycle and the level of lipid present in the coral tissue. Simpson (1987) notes that some coral species experience a decrease in total lipid after planulation and after drastic, rapid changes in the level of available sunlight. These findings are consistent with Patton et al. (1977) and Crossland et al.'s (1980) suggestions that excess lipid is stored as an energy reserve for use during periods of reproductive activity and environmental stress.

The use of the ratio of storage to structural lipids provides insight about coral condition without the confounding effects of sampling error associated with determination of total lipid. Under non-stressed conditions, extremely high storage levels of wax ester and triacylglyceride are maintained in *Montastrea annularis* and *M. faveolata* by over-production of fixed carbon by the zooxanthellae (Patton et al., 1977,

Stimpson, 1987, Harland et al., 1992). Lipid production and use are typically balanced by normal metabolic functions (Ducklow and Mitchell, 1979, Ward, 1995), growth (Stimpson, 1987, Fang et al. 1994), energy storage for reproduction and planulation (Ward, 1995, Battey et al., 1998) and loss as coral mucus (Patton et al., 1977. Crossland et al., 1983, Stimpson, 1987, Harland et al., 1993, Ferrier-Paget et al., 1998a). These stored lipids are produced and exploited rapidly with replacement occurring rapidly after depletion in non-stressed colonies (Crossland et al., 1980, Stimpson, 1987, Battey et al., 1998, Ferrier-Paget et al., 1998a). However, environmental conditions associated with sediment stress can slow this replacement.

Under sediment stress, coral colonies receive decreased light levels (Stimpson, 1987, Rogers, 1990, Telesnicki and Goldberg, 1995), incur increased metabolic demand (Abdel-Salam et al. 1988, Telesnicki and Goldberg, 1995) and suffer additional loss of lipid due to increased production of mucus for sediment clearing (Crossland et al., 1983, Stafford-Smith and Ormond, 1992, Stafford-Smith, 1993). The effects of sedimentation on scleractinians are manifest as decreased net photosynthesis (Abdel-Salam et al., 1993, Telesnicki and Goldberg, 1995) and an increase in use and loss of storage lipids which results in a net decrease in the levels of stored lipids in sediment-stressed corals. Therefore, given low levels of sedimentation and low wave energies, with no visible tissue damage and no change in the levels of structural lipids, the levels of storage lipids decreased in the affected colonies. This net loss of storage lipid results in the reduction of storage to structural lipid ratio found in the study colonies. Given this reduction in lipid ratio, questions remain as to whether this loss of storage lipids is due to reduced

photosynthetic production or increased host metabolism or a combination of both processes.

Fatty Acid Composition

Comparison of the fatty acid profiles for the control and experimental colonies provides insight into the nature of the symbiotic relationship under normal and sediment stressed conditions. The relative abundance of PUFA in coral has been found to vary widely (Meyers, 1979, Latyshev et al., 1991, Harland et al., 1993) and may be an artifact of the lipid analysis technique. The results of this study coincide with Latyshev et al. (1991) indicating that the majority of the polyunsaturated fatty acids (PUFA) are located in the polar lipid fraction of the sample. Yet, in an analysis of Total Lipid Fatty Acids, Meyers (1979) reported that 22 out of 28 species contained < 5% PUFA. These results are not extraordinary considering the low ratio of polar lipids to neutral lipids found in healthy coral (Patton et al., 1977, Harland et al., 1993). Harland et al. (1993) point out that Meyer's work is contradicted by Latyshev et al. (1991) who identified up to 60% PUFA in the polar lipid fraction of Pacific corals.

This difference in PUFA abundance may then be attributed to the separation of lipids into neutral and polar fractions prior to analysis. Harland et al., (1993) found *Montastrea annularis* contained 9.9 % PUFA when fatty acid analysis was conducted on total lipid and give relative abundances of WE and TAG for *Montasrea annularis* in the above study which comprise 65% of the total lipid. This indicates a neutral lipid to polar

lipid ratio of 65:35 for the study colonies. However, the majority of the PUFA are located in the polar fraction. Adjusting for relative levels of neutral and polar lipids we can calculate the approximate amount of PUFA in the polar fraction using the formula:

$$(\%\ \text{PUFA in lipid fraction}) = \frac{(\%\ \text{PUFA in Total Lipid})}{(\%\ \text{Total lipid for polar fraction})}$$

Given the quantities Harland et al. (1993) present, we can estimate that ≈ 33 % of the polar lipid fraction would be PUFA, which is consistent with the results of the present study. Further study of PUFA in coral should examine *both neutral and polar fractions, rather than total lipid, to eliminate the effects of large quantities of saturated, neutral, stored lipid on the identification and abundance of PUFA.*

Differences in fatty acid composition of experimental versus control colonies suggest a net decrease in zooxanthellar membrane in the sediment-stressed coral. The coral/algal symbiosis has been shown to rapidly store fixed carbon as membrane, which primarily consist of the (polar) phospholipids (Gattuso et al., 1993). The decreased levels of the plant and dinoflagellate PUFA (18:3 and 18:4) in the polar lipids suggest the relative loss of membrane containing these fatty acids. The decrease in algal membrane may result from a net decrease in the number of chloroplasts which are 35% lipid and comprise up to 75 % of the membrane of the zooxanthellae (Darnell et al., 1990) or may result from a expulsion of zooxanthellae from the tissue. This investigation intimates that low-level sedimentation stress results in "bleaching" of coral tissue and warrants further investigation.

Recent work by Ferrier-Pagét et al., (1998b) implies a nutritional benefit of mucus production in scleractinian corals through increased prey capture. Whether increased mucus present for sediment clearing provided a nutritional benefit, via increased feeding, was not evident in the fatty acid composition of the stressed colonies, but bears further investigation.

Lipids and Environmental Stress

In their summaries of the effects of sedimentation effects on hard coral Rogers (1990) and Brown and Howard (1985) address the tremendous range, and contradictory outcomes of coral response to sediment stress. Brown and Howard (1985) provide numerous examples of resistance to, and recovery from, severe sedimentation in both the field and laboratory while other studies indicate that small changes in sediment regime result in the destruction of healthy reefs (Loya, 1976, Hands et al., 1993, van Katwijk et al., 1993).

This apparent "paradox" of effects may result from a failure to adequately reflect matters of scale when considering changes in reef community structure (Harriott, 1994). Most experimental investigations are performed on the individual colony, organism-level over relatively short time scales (hours to weeks), while most analysis of coral reef community change (percent live cover and species diversity) can only be recognized over relatively long time scales (years to decades). As suggested by Harriott (1994), the inconsistencies in response to stress from the organism to the community level clearly suggest a need to develop indicators of coral condition which can link change at the

organism level with changes at the community level. Biochemical indices may provide the tools to evaluate short-term changes in coral condition which are also relevant to long-term changes in condition at the individual and community levels.

The ratio of structural lipids to storage lipids in coral appears to be an indicator of coral condition in *Montastrea spp.*. Lipid ratio is a tool that can be measured on the colony scale, but has ramifications on the community level and could be affective in monitoring sub-lethal stresses in coral. This study found that colonies of *Montastrea faveolata* growing in the near-pristine, deep-water environment surrounding the CMRC research station at Lee Stocking Island, Bahamas, maintained ratios of storage to structural lipid (≈2.0) which were consistent with previously published data (Harland et al. 1993) during non-sedimentation conditions. These levels dropped significantly after sedimentation and returned to baseline levels within 3 days of the sedimentation event. The colonies of *Montastrea annularis* growing in shallow, near-shore, patch reefs exhibited a much lower baseline ratio (< 0.35) of storage to structural lipids. The lipid ratio of the non-stressed (and recovered) colonies of *Montastrea faveolata* in this study compares with Harland et al.'s (1992, 1993) lipid levels for *Montastrea annularis* for non-stressed colonies suggesting no difference in lipid ratio due to species differences. *Montastrea spp.* have been shown to maintain equivalent lipid levels despite increased depth and decreasing light levels through an unidentified compensation mechanism (Meyers et al., 1979, Harland et al., 1993). These depth data imply that differences in colony depth between the two study sites should not affect baseline nutritional state of the sample colonies. While the low ratio of storage to structural lipids may have resulted

from differences in the preservation techniques employed in this study, Harland et al. (1993) suggest that the method of sample preparation only minimally effects recovery of lipid. This evidence supports the discounting of sample preparation as a source of the differences in baseline lipid ratio.

The differences in baseline lipid ratio between these shallow-water *Montastrea annularis* colonies and the deep-water *Montastrea faveolata* colonies may result from differences in metabolism at the coral and/or symbiont level, differences in sample preservation and storage protocol, or from differences in local environment. The effects of chronic sedimentation, run-off and resulting increased water temperature have been shown to limit coral growth and diversity on the coasts of the Puerto Rican islands (Kaye, 1959, Almy and Carrion-Torres, 1963, Loya, 1976). Additionally, the Vieques sediments were shown to contain measurable silts and were primarily comprised of terrigenous sediments. In addition to the chronic sedimentation reported for the northwest coastal areas of Puerto Rico, water temperatures at the Vieques study site exceeded 29.0° C during the experimental period. While none of the experimental or control colonies showed any signs of bleaching during this study, elevated water temperatures have been shown to reduce photosynthetic activity in *Montastrea annularis* (Goreau and MacFarlane, 1990) which contributes to the effects of reduced growth rate and limited reproductive success. Reduced growth rate and reproductive success are also directly linked to nutritional condition and may be evident through the presence of available storage lipids. This study suggests that local environmental conditions may constitute a great influence on the typical, baseline nutritional state of the reef coral present. The

results of this study further suggest that lipid ratio can be considered a bio-indicator of the health of individual colonies of *Montastrea* over short (24 hours) time scales and may also be a useful tool to determine individual and community level change on meso (weeks/months/years) time scales.

The analysis of tissue lipid ratio may be a useful tool in coral research and resource management. The TLC/FID equipment is relatively inexpensive (<$20,000) and simple, qualitative analysis are easily obtained and processed, enabling resource management agencies to monitor coral condition on short-time scales with a minimum of expense and training. However, the rigorous, quantitative examination of tissue lipid subclass can also be conducted by experienced biochemical researchers to measure coral stress response on short time scales.

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CHAPER III

ENVIRONMENTAL STRESS AND HARD CORALS: EVALUATING LIPID AS AN INDICATOR OF HEAT STRESS IN THE HARD CORAL *MONTASTREA ANNULARIS*

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ABSTRACT

This study examines the changes in lipid subclass of *Montastrea annularis* subject to heat-induced bleaching to determine possible linkages between the loss of photosynthetic pigments and the nutritional condition (growth and reproductive potential) of the host coral. The level of storage lipid (wax esters and triacylglycerides), structural lipid (sterol esters and phospholipid) and Free Fatty Acid (FFA) were measured in colonies of *Montastrea annularis* subject to heat stress. Experimental colonies were exposed to 35°C until bleaching was visually evident in all colonies. Ratio of storage lipids to structural lipids did not change with heat treatment, but levels of FFA increased in treatment colonies from a mean level of 0.012 ($n = 22$, s.e. = 0.003) to a level of 0.156 ($n = 22$, s.e. = 0.01) which was significant ($p < 0.05$). Fatty acids of the treated colonies showed a dramatic change in the polar fraction, with significant decreases in the18:3(n-6), 18:3(n-3), 18:4(n-3), 20:4(n-6) and 20:5(n-3) polyunsaturated fatty acids (PUFA) and subsequent significant increases in the saturated fatty acids, 16:0 and 18:0. The elevated levels of FFA in the tissue lipids, along with the dramatic decrease in polar lipid PUFA support previous studies which showed that corals subject to severe heat stress digest algal symbionts as a reaction to the stress.

INTRODUCTION

Coral bleaching, the expulsion of algal symbionts (zooxanthellae) or the reduction and loss of photosynthetic pigment, is a well-documented stress response in scleractinian coral and the wide-spread occurrence of coral bleaching continues to capture the attention of scientists, resource managers and other environmental groups. Rarely, conditions of extreme starvation (Yonge and Nichols, 1931) and sedimentation (Rogers, 1983, 1990) have been shown to induce bleaching. However, mass bleaching during the last decade has more commonly resulted from small changes in water temperature above mean summer levels (Glynn and D'Croz, 1990, Jokiel and Coles, 1990, Fitt et al., 1993, Fitt and Warner, 1995, Lesser, 1997), exposure to high levels of ultraviolet radiation (Lesser, et al., 1990, Kinzie, 1993) or the synergistic association of these two factors which cause the most profound, and widespread, bleaching events (Jaap, 1979, Fisk and Done, 1985, Cook et al., 1990, Glynn, 1991, Fitt et al., 1993).

Hard coral possess a variety of responses to countermand the effects of heat stress (Fitt and Warner, 1995). As elevated temperatures persist from hours to days in duration, coral responses range from reducing photosynthetic pigments within individual zooxanthellae (Hoegh-Guldberg and Smith, 1989, Fitt and Warner, 1995) to the complete expulsion of algal symbionts (Jaap, 1979, Lesser, 1997). Mechanisms controlling these responses are not well understood (Fang et al., 1997) but recent studies indicate that bleaching may be triggered by oxidative stress due to overproduction by the zooxanthellae (Lesser et al., 1990, Lesser, 1997) initiated by a disruption of the dark phase of photosynthesis (Jones et al., 1998). Regardless of the cellular changes which

result in bleaching, sub-lethal effects of the condition include a net decrease in photosynthetic potential (Fitt and Warner, 1995) and a reduction in the photosynthesis to respiration (P:R) ratio; with abnormally high respiration (> 9 days) after temperatures return to normal (Hoegh-Guldberg and Smith, 1989, Fitt and Warner, 1995). Longer-term effects of bleaching include decreased growth rate (Goreau and MacFarlane, 1990, Leder et al., 1991), a decreased ability to regenerate damaged tissue (Meesters and Bak, 1993) and decreased reproductive potential (Szmant and Gassman, 1990). These effects, which can persist for several months after the corals begin to restore zooxanthellae to normal levels, indicate a correlation between the presence of adequate zooxanthellae and coral growth, tissue maintenance and reproduction.

The symbiotic relationship between the host and algal symbionts under normal, non-stressed conditions is highly productive, enabling host tissue to accumulate large quantities of adequate storage lipid through translocation of fixed carbon from photosynthesis (Patton et al., 1977, Harland et al., 1993). The zooxanthellae translocate up to 90% of daily fixed carbon to the host tissue (Crossland et al., 1980, Muscatine et al., 1984) which appears to constitute overproduction of lipid (Stimpson, 1987). This rapid production, translocation and storage of fixed carbon as lipid may be necessary due to the rapid use and loss of lipid by coral tissues (Stimpson, 1987). In addition to requirements of normal metabolism and reproduction, corals lose up to 50% of excess lipid in mucus daily (Crossland et al., 1980, Muscatine et al., 1984, Battey et al., 1998). The maintenance of large quanities of stored lipid may enable coral to survive stress events with minimal disruption of the symbiotic relationship (Patton et al. 1977), yet

turnover of lipid is rapid (Patton et al. 1977, Crossland et al., 1980, Muscatine et al., 1984, Battey et al., 1998). The symbiotic relationship may therefore become quickly compromised under stressed conditions. The lost, or decreased ability to gain fixed carbon from zooxanthellae during bleaching may result in the decreased growth, tissue maintenance and reproductive abilities and may be evident as a function of the amount of storage lipids available for use during the stressful event.

Reef-building corals store nutrient reserves as wax esters (WE) and triacylglycerides (TAG)(Patton et al., 1977, Harland et al., 1992). Levels of WE and TAG can fluctuate with coral reproductive state (Stimpson, 1987), productivity of algal symbionts (Patton et al., 1977, Stimpson, 1987), metabolic requirements and food availability (Crossland et al., 1980, Crossland, 1987). The sterol esters (SE, primarily cholesterol) and the polar lipids (including the phospholipids, PL) are structural lipids and remain relatively stable with short-term changes in nutritional state (Meyers, 1979). Changes in lipid class abundance will be manifest in the storage lipid (through use or decreased production of photosynthate) while the levels of structural lipids should remain constant on short time scales. These fluctuations in lipid class levels provide the basis for evaluation of lipid ratio as an indicator of coral condition given the previously reported coral responses to sedimentation stress. Lipid ratio has been used to identify changes in the nutritional condition of *Montastrea annularis* and *M. faveolata* during periods of experimentally- and naturally-induced sedimentation stress (Chapter 2).

Analysis of the lipid sub-units, fatty acids, may provide insight into the relative contribution of zooxanthellae and host tissue in response to sub-lethal stress. Fatty acids consist of long carbon chains that maintain their structural integrity through trophic levels. This structural integrity allows fatty acids to be used as biomarkers in feeding studies. For example, only plants can produce linolenic acid (18:3n-3) and linoleic acid (18:2n-6) and large amounts of PUFA are indicative of marine plants and dinoflagellates (Meyers et al, 1978, Meyers, 1979, Harland et al., 1993). Little data has been collected on coral fatty acids and the relative abundance of saturated fatty acids (no double bonds between carbon atoms), unsaturated fatty acids (one double bond) and polyunsaturated fatty acids (PUFA, ≥ 2 double bonds) remains unresolved (Harland et al., 1993). However, the saturated fatty acids, palmitic acid (16:0) and stearic acid (18:0) are the primary fatty acids in coral (Meyers, 1979) and recent research by Al-Moghrabi et al. (1995) indicate the importance of arachidonic acid (20:4 n-6) and docosatrienoic (22:3 n-3) PUFA in corals subject to stress.

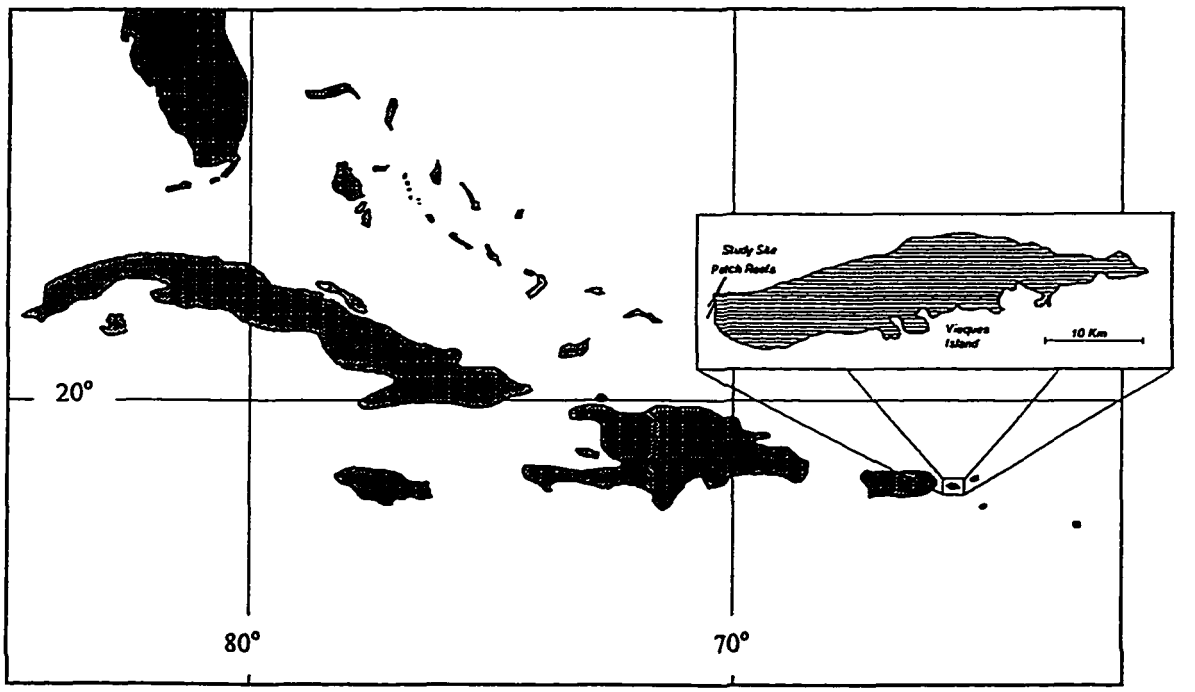
This study will examine changes in tissue lipids of *Montastrea annularis* subject to heat stress to evaluate the role of bleaching on tissue energy reserves, and the change in tissue lipid subclass ratio after experimental heat stress. The lipid subclass ratio was further evaluated for consistency and accuracy to assess the usefulness of this method in monitoring coral on short time scales. Analysis of fatty acid composition was completed to investigate the relative changes in algal- and host-derived fatty acids in corals subject to heat stress.

MATERIALS AND METHODS

Experimental Heat Stress

Ten colonies of *Montastrea annularis* were haphazardly selected from small, leeward patch reefs (depth = 3.0 m) adjacent to the northwest coast of Vieques, Puerto Rico (18°06'34" N, 65°34'32"W)(Figure 5) and marked for use (5 ambient and 5 treatment) in the heat stress experiments from July 14-19, 1997. Tissue samples were collected by a SCUBA diver using a geologist's pick, by removing a small (3.0–8.0) cm^2 section of tissue and skeleton from each colony. Each section of coral was individually placed in a Whirlpak® collection bag containing seawater and stored in a cooler for return to the laboratory. Ambient colonies (n=5) were left *in situ* while the five experimental colonies were removed with a geologist's hammer, returned to the lab and placed in a large aquarium of locally collected seawater. Ambient colonies were not removed to aquaria as true control colonies because of permitting restrictions and limited laboratory resources. The aquarium containing the experimental colonies was kept indoors, aerated, and shaded from direct sunlight throughout the experiment. Water temperature was increased to 35°C using aquarium heaters. Experimental corals were removed and preserved at first incident of bleaching (35 hours). Bleaching was defined as an identifiable loss of pigment, determined by visual examination. Upon return to the laboratory each sample was individually preserved in a solution of 10% Formalin and filtered seawater (1/10 v/v) for 24 hours. After preservation, the sample was rinsed in filtered seawater and frozen, at –20°C, for transfer, on ice, to the Virginia Institute of Marine Science. Samples were stored at –20°C until processing.

Figure 5. Location of the study site at Vieques, Puerto Rico.



Five replicates of coral tissue (3-5 adjacent polyps) were removed from the carbonate skeleton of each of the colonies with fine forceps, placed in pre-weighed amber collection vials, and freeze-dried in a VIGRIS (Gardiner, NY) desiccator/freezer. The vials were re-weighed to obtain a dry weight of the tissues and remained frozen until processing.

Lipid Class Analysis

Lipid class content was determined using an Iatroscan thin layer chromatograph (TLC) with a flame ionization detection (FID) system (Iatroscan Laboratories Inc., Tokyo Japan). Coral tissue lipids were extracted twice from homogenized polyp tissues using modified methods of Folch et al. (1957) and Bligh and Dyer (1959). Briefly, this procedure separates and extracts lipids from tissue in a biphasic solution of chloroform/methanol/water (2:2:1, v/v). Stearyl alcohol was added to the samples prior to extraction as an internal standard to correct for extraction efficiency. Approximately 10-25 mg of dry sample was extracted for lipid class analysis. The lipid phase was captured, pooled for the two extractions, and evaporated to dryness to concentrate the sample to 100 µl. Two (2) µl of concentrated lipid extract were spotted on S-III chromarods and lipid classes were developed in hexane:diethyl ether:water (85:15:0.04) to separate the lipid classes. The chromarods were scanned and lipid classes were quantified by standard curve on using a Mark III Iatroscan analyzer.

Fatty Acid Methyl Esterization (FAME)

Tissue lipids were extracted as described above. Lipids were re-extracted in a solution of DCM:MeOH (98:2). Neutral lipids (wax ester, triacylglyceride, sterol esters) were separated from polar lipids (phospholipid) using a silica gel micro-column and rinsed to 13 ml with DCM:MeOH (98:2) and MeOH respectively. Each fraction was collected in a graduated vial containing 5.0 µl of C 23:0 as an internal standard. Methyl esterization of the fatty acids was accomplished with Boron trifloride (BF_3). The FAMEs were extracted and cleaned with carbon disulphide (CS_2) and water. Samples (5.0 µl) were analyzed using a VARIAN 3300 Gas Chromatograph (Varian Inc., Walnut Creek, CA) coupled with a flame ionization detector. FAMES were identified determined using comparisons to retention time and quantities of internal standards.

Data analysis

Coral tissue lipid responses to heat stress were measured and presented as the ratio of storage lipid to structural lipid (unless otherwise noted) and statistical tests were made using arcsine transformed proportions. Statistical comparisons of experimental results are, unless otherwise noted, Student's *t*-tests (two-tailed) of pre- and post-treatment coral colonies. The use of individual tissue samples, taken from the same colony, as independent samples has been widely used and tested (Meyers et al., 1978, Meyers, 1979, Latyshev et al., 1991, Harland et al, 1992, Kinzie, 1993).

RESULTS

Corals subject to experimental condition experienced an increase in temperature (32-35° C) until bleaching was observed in each colony (35 hours). The maximum treatment temperature was 35.2 ° C which was 5 ° C higher than the mid-day (1200) temperatures recorded at the collection site (29.4 ° C). Experimental colonies did not exhibit tissue sloughing and did not appear dead at the time of removal and preservation despite the severe temperatures, a result in contrast to Fitt and Warner (1995) who describe mortality in *Montastrea annularis* after 19 hours at 34 ° C.

Lipid ratio

Lipid class composition (mg lipid/g dry tissue) for ambient and experimental colonies are presented in Table 7. Level of total lipid did not change significantly with treatment (Figure 6). The ratio of storage (WE+TAG) to structural (SE+PL) lipids did not change significantly in corals subject to heat stress despite bleaching of the treatment colonies (Figure 7a). The ratio of SE+PL:Total lipid fluctuated in both the ambient colonies and in the experimental colonies but did not change significantly with treatment (Figure 7b). The level of FFA in the tissues of the heat-stressed colonies (n = 22, mean = 2.85 mg/g dry tissue, s.e.= 0.41) represented an increase of an order of magnitude over pre-treatment (n = 22, mean = 0.36 mg/g, s.e. = 0.08) and ambient samples, with a representative increase in FFA:Total lipid ratio of 0.01 (s.e. = 0.001) to a post-treatment level of 0.16 (s.e. = 0.01)(Figure 7c).

Table 7. Lipid subclass composition (mg lipid/g dry tissue) for pre- and post-treatment colonies of *Montastrea annularis* subject to heat stress.

	Pre-treatment				Post-treatment			
	Lipid	s.e.	%	s.e.	Lipid	s.e.	%	s.e.
WE	3.74	0.32	11.70	0.82	1.54	0.17	9.65	1.10
TAG	2.02	0.24	6.33	0.58	0.85	0.12	4.67	0.54
Total Storage Lipid	5.75	0.42			2.39	0.28		
FFA	0.36	0.08	1.18	0.30	2.85	0.41	15.60	1.18
SE	4.01	0.38	12.20	0.24	1.15	0.16	6.04	0.54
PL	22.60	2.13	68.60	1.05	15.70	3.16	64.10	2.53
Total Structural Lipid	26.66	2.49			16.82	3.30		
Total Lipid	31.40	2.98			22.06	3.86		
FFA:Total	0.01	0.00			0.16	0.01		
(WE+TAG):(SE+PL)	0.23	0.01			0.22	0.03		
(SE+PL):Total	0.81	0.01			0.70	0.02		

Figure 6. Mean pre- and post-treatment levels of total lipid (mg lipid/g freeze dried tissue weight ± standard error) for colonies of *Montastrea annularis* subject to 35°C until bleached (two-tailed *t*-test, $p > 0.15$). Ambient ■ colonies remained *in situ* while experimental ▨ colonies were treated in the laboratory.

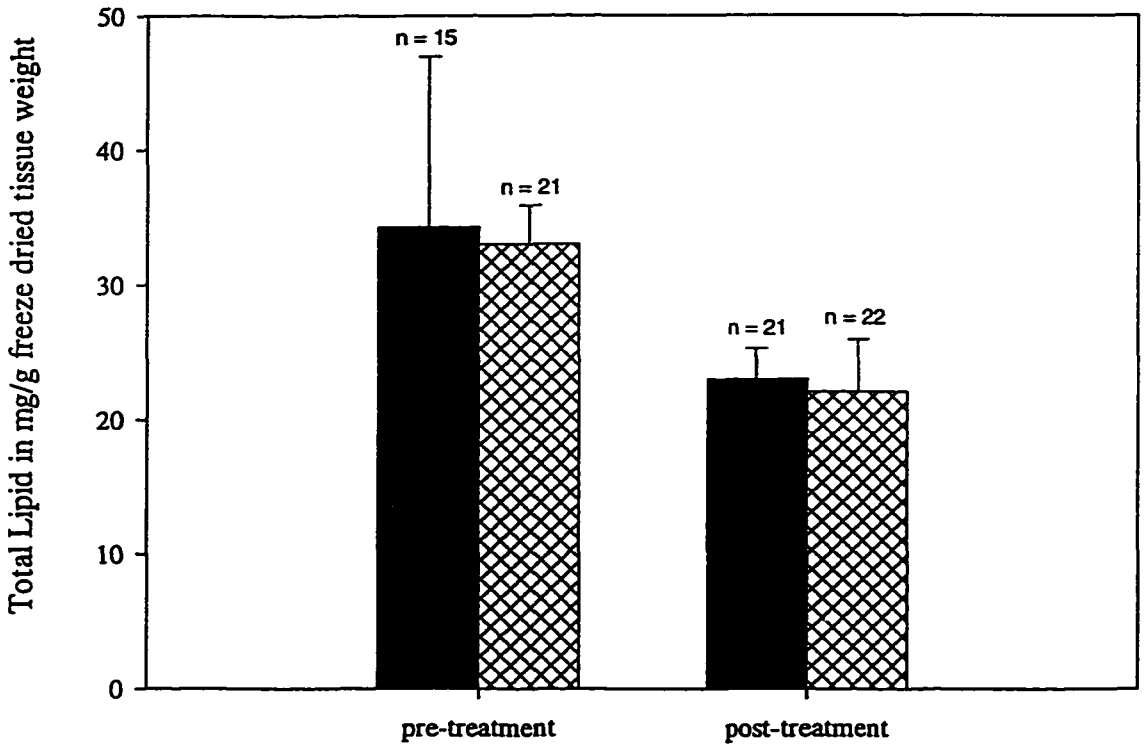


Figure 7. Mean ratio (± standard error) of lipid subclass of *Montastrea annularis* subject to heat stress in between (●) ambient and (▲) experimental colonies. (A) Mean ratio of structural lipid to total lipid (SE+PL:TOTAL LIPID) for pre- and post-treatment condition with no significant differences between pre- and post treatment ratio (two-tailed *t*-test, $p > 0.25$). B. Storage lipid to structural lipid (WE+TAG:SE+PL) with no significant differences between pre- and post treatment ratio (two-tailed *t*-test, $p > 0.25$). (C) Free Fatty Acid:Total lipid (FFA:TOTAL LIPID) increased significantly in experimental colonies pretreatment levels (two-tailed *t*-test, $p < 0.01$).

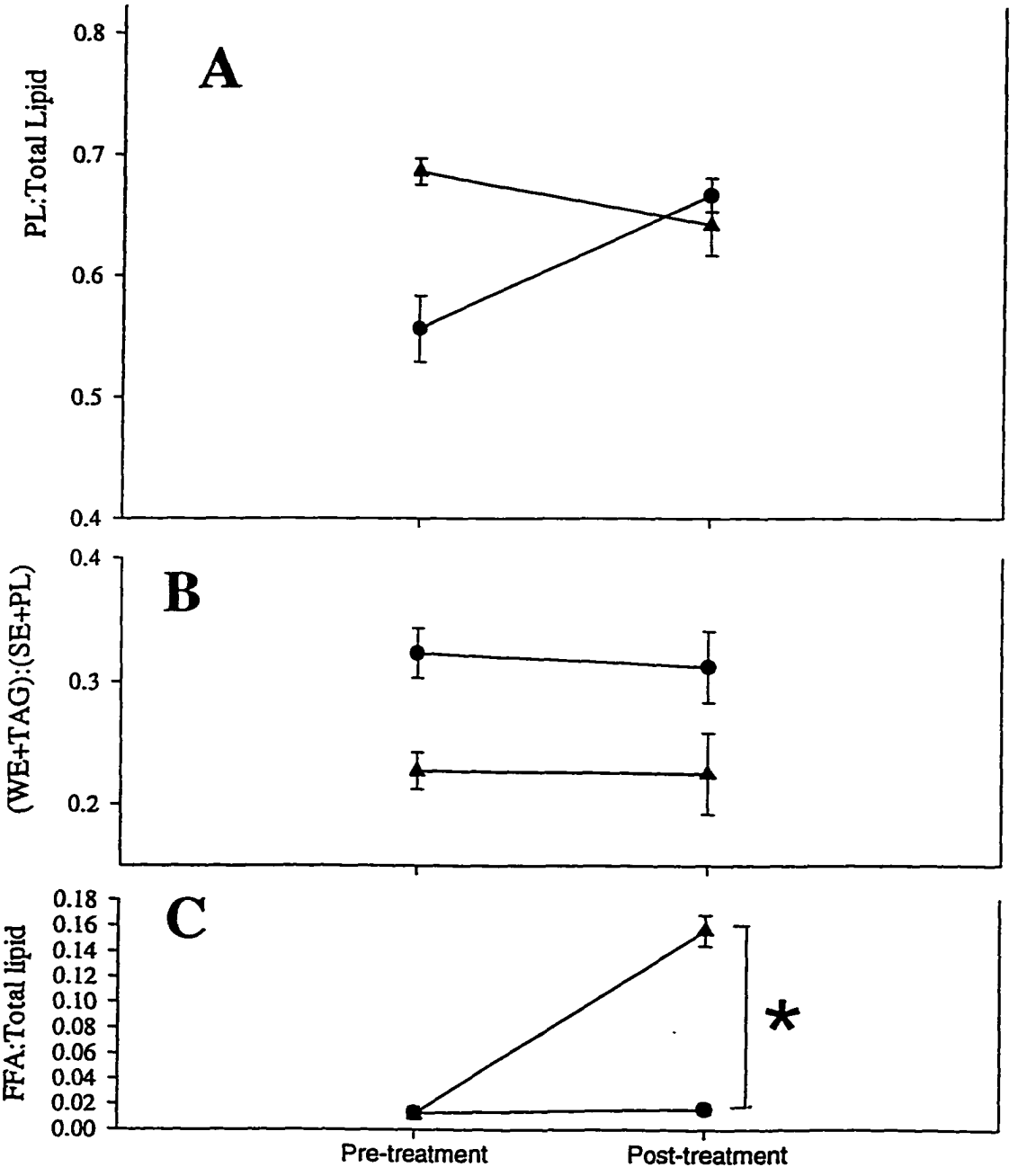
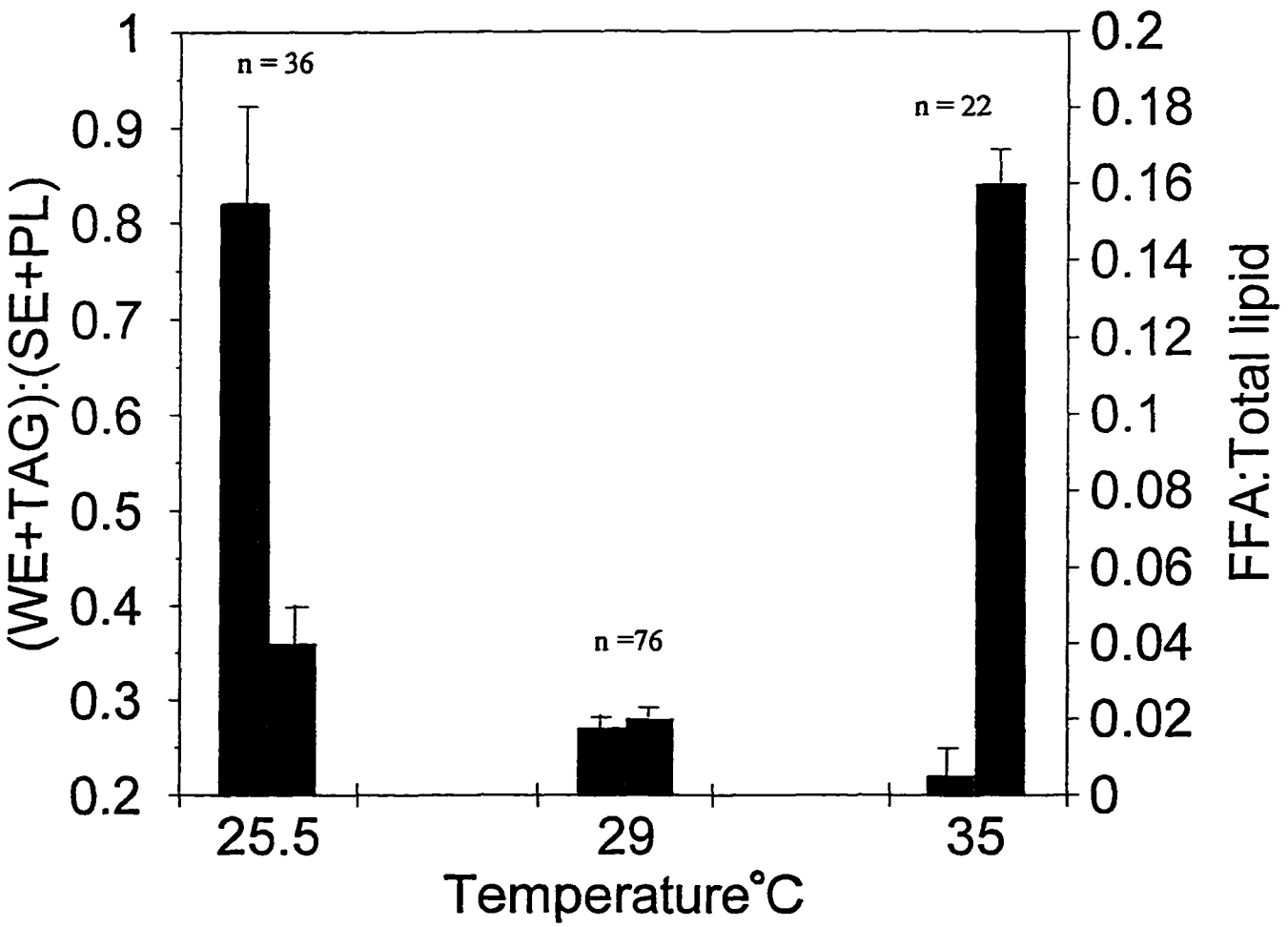


Table 8. Mean (n = 4) weight percent fatty acid composition (% Total lipid) of the neutral and polar lipid fractions of *Montastrea annularis* subject to two levels of heat stress, Ambient (29°C) and Experimental (35°C). Significant differences are denoted by (*) (two-tailed *t*-test, $p < 0.05$). Notation shows the number of carbon atoms, followed by number of double bonds and the position of the terminal double bond.

	Neutral Lipids				Polar Lipids				Harland, et al. (1993)
	Control		Heat		Control		Heat		Total Lipid
		(S.D.)		(S.D.)		(S.D)		(S.D.)	
14:0	3.16	(0.54)	3.79	(1.13)	1.43	(0.68)	3.27	(1.64)	
15:0			0.15	(0.03)	15.04	(2.77)	0.14	(0.23)	1.10
16:0	59.24	(1.62)	56.51	(8.04)	0.29	(0.19)	40.41*	(11.61)	58.60
17:0	0.15	(0.09)	0.13	(0.13)	9.16	(1.82)	0.52	(0.69)	
18:0	9.98	(0.96)	11.27	(1.79)	0.87	(0.13)	18.04*	(5.45)	12.40
19:0									3.30
16:1(n-7)	2.10	(0.19)	1.38	(0.90)	1.73	(0.39)	0.42	(0.36)	1.90
18:1(n-9)	5.42	(0.88)	5.01	(1.47)	0.99	(0.23)	1.67	(0.61)	7.10
18:1(n-7)	1.19	(0.67)	0.58	(0.92)	0.00	(0.00)	0.34	(0.34)	1.40
20:1(n-11)	0.00	(0.00)	0.00	(0.00)	0.91	(0.54)	0.00	(0.00)	
20:1(n-9)	1.16	(0.18)	0.98	(0.54)	0.00	(0.00)	0.73	(0.96)	1.90
20:1(n-7)	0.00	(0.00)	0.00	(0.00)			0.00	(0.00)	0.20
16:2(n-7)	0.37	(0.10)	0.18	(0.13)	0.60	(0.54)	0.06	(0.11)	
16:2(n-4)	0.12	(0.21)	0.73	(0.78)	0.98	(0.54)	0.99	(0.63)	
16:3(n-4)	0.00	(0.00)	0.00	(0.00)	0.27	(0.28)	0.00	(0.00)	
16:4(n-3)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.11	(0.15)	
18:2(n-6)	1.30	(0.15)	1.30	(0.13)	1.49	(0.42)	1.41	(0.54)	1.20
18:3(n-6)	2.25	(0.17)	1.62	(0.57)	8.68	(1.06)	3.14*	(1.01)	
18:3(n-3)	0.00	(0.00)	0.04	(0.06)	2.82	(0.99)	0.81*	(0.81)	0.20
18:4(n-3)	0.42	(0.09)	0.68	(0.61)	12.06	(1.81)	3.08*	(1.15)	
18:5(n-3)	0.25	(0.15)	0.29	(0.38)	7.81	(1.11)	1.50	(1.39)	
20:2(n-6)	0.42	(0.20)	0.77	(0.62)	0.81	(0.74)	0.80	(1.02)	0.30
20:3(n-6)	0.84	(0.09)	0.34	(0.34)	0.23	(0.23)	0.00	(0.00)	1.40
20:4(n-6)	2.42	(0.29)	4.22	(3.14)	12.01	(0.76)	5.83*	(2.89)	2.20
20:5(n-3)	0.97	(0.15)	0.75	(0.51)	3.88	(1.06)	1.59*	(0.96)	0.50
21:5(n-3)	0.02	(0.04)	0.00	(0.00)	0.80	(0.12)	0.00	(0.00)	
22:4(n-6)	0.81	(0.20)	0.15	(0.15)	3.21	(0.21)	2.09	(0.84)	
22:5(n-6)	0.00	(0.00)	2.06	(1.82)	0.00	(0.00)	1.83	(0.73)	0.90
22:5(n-3)	0.26	(0.07)	0.20	(0.20)	0.72	(0.17)	0.18	(0.30)	0.30
22:6(n-3)	4.88	(0.24)	2.07	(1.33)	6.66	(0.21)	4.68	(2.79)	2.90
TO.SAT.	73.42	(1.04)	72.48	(9.51)	26.19	(4.97)	63.35	(14.10)	
TO.MONO	10.10	(1.56)			4.65	(0.92)	3.55	(0.89)	13.00
TO.POLY	15.35	(0.47)	8.02	(3.49)	63.11	(4.61)	28.10	(9.97)	9.90
(n-3)/(n-6)	0.82	(0.07)	0.35	(0.09)	0.99	(0.07)	0.78	(0.23)	
22:6/20:5	5.17	(0.99)			1.87	(0.56)	3.39	(1.37)	
22:5/20:4	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	
TOTAL	97.74	(0.00)	100.00	(0.00)	93.45	(0.00)	100.00	(0.00)	99.10

Figure 8. Mean ratio of □ (WE+TAG):(SE+PL) and ratio of ■ FFA:Total lipid in colonies of *M. annularis* collected at Vieques, Puerto Rico plotted against water temperature. 29°C and 35 °C samples are from the present study while the 25.5 °C samples were collected at the same location in April, 1998.



Fatty Acid Composition

Results of the FAME analysis are found in Table 8. The neutral lipids were predominantly composed of saturated fatty acids and 16:0 and 18:0 saturated fatty acids constituted between 69-73% of the total fatty acids, with only small amounts of PUFA. There were no significant differences in neutral lipid fatty acids between ambient and heat-stressed tissues. The polar lipid contained < 60% PUFA, including the algal-derived 18:3(n-3) and 18:4(n-3) fatty acids. Experimental colonies exhibited a significantly lower level of PUFA 18:3(n-6), 18:3(n-3), 18:4(n-3), 20:4(n-6) and 20:5(n-3)(two-tailed *t*-test, $p < 0.05$) and a significantly higher level of the saturated fatty acids 16:0 and 18:0 (two-tailed *t*-test, $p < 0.05$) than ambient colonies.

DISCUSSION

The relative levels of storage (WE+TAG) to structural (SE+PL) did not change significantly in bleached colonies of *Montastrea annularis* subject to severe, sudden heat-stress. This contrasts with the response of *Montastrea annularis* to minor, environmental stress (sediment stress) despite the net decrease in zooxanthellar membrane tissue in both instances (Chapter 2). Moderate sub-lethal increases in water temperature have been shown to reduce photosynthesis, limit growth and reduce coral ability to repair and regenerate tissue (Szmant and Gassman, 1990; Fitt and Warner, 1995). Photosynthesis, growth and basic metabolism are linked to coral nutritional state and may relate to levels of stored lipid. Moderate increases in water temperature appear to affect the relative amounts of stored lipid, and general nutritional condition of *Montastrea annularis* (Figure 8).

This study indicates that as water temperature approaches lethal limits, the ratio of storage to structural lipid ceases to decrease and the relative amount of FFA increases, intimating a change in metabolic strategy. While some of the increase may result from peroxidation of tissue lipids due to increased sample temperatures, an increase in FFA also indicates an increase in lipid metabolism. This increase in lipid metabolism in heat-stressed cells is supported by the work of Fang et al. (1997) who report increased levels of tissue Ca^{2+} which is indicative of the enzymatic processes of exocytosis in binding and releasing lipases and maintaining proper cellular pH in the lysosomes. Suharsono et al.

(1993) further support these results and suggest increased lysosomal activity, associated with the zooxanthellar membranes, in anthozoans subject to heat stress.

Changes in fatty acid composition of the heat-stressed colonies suggest that the metabolism of lipid membranes is preferentially targeted at algal tissues, showing a dramatic decrease in 18:3(n-6), 18:3(n-3) and 18:4(n-3) and the subsequent, relative increase of the saturated fatty acids 16:0 and 18:0 in the polar fraction. This postulated reduction of the net level of algal membrane supports previous research which suggests that the catabolism of zooxanthellae is one mechanism to reduce the number of zooxanthellae under heat-stressed conditions (Hoegh-Guldberg et al., 1989, Szmant and Gassman, 1990, Suharsono et al., 1998).

Coral subject to environmental stress use stored lipids to maintain the symbiotic balance during stressful periods (Crossland et al. 1980, Crossland, 1987, Stimpson, 1987, Chapter 2) The selective use of storage lipids has been shown to occur in *Montastrea annularis* and *Montastrea faveolata* under conditions of sedimentation stress (Chapter 2). The severe, heat-stressed conditions present in this study appear to induce an alternate strategy for coping with stress. Corals subject to lethal stress do not appear to use stored lipids in an attempt to maintain a static state, but immediately begin to reduce zooxanthellae density including the digestion of zooxanthellae. Fitt and Warner (1993) report that the process of bleaching is rapidly increased in *Montastrea annularis* as the temperature approaches lethal levels and the corals begin to cease metabolic processes, but this study intimates two distinct strategies for coping with stress. These processes can

be supported by changes in fatty acid composition and can be identified by changes in the ratios of various lipid subclasses. The dramatic decrease in the ratio of storage to structural lipid indicates a maintenance strategy (Chapter 2) for sub-lethal, low-level sedimentation stress, while the dramatic increase in the ratio of FFA to Total lipids indicates a severe reaction, with tissue degradation to the presence of lethal, heat stress.

The evaluation of the ratio of FFA to Total lipids may provide early indication of corals under lethal stress and may provide insights into environmental conditions which result in acute, coral death. This study indicates that the analysis of coral lipid ratio under changing environmental, metabolic conditions bears further investigation.

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CHAPTER IV

ENVIRONMENTAL STRESS AND HARD CORAL: EVALUATING A TECHNIQUE FOR MONITORING CORAL LIPID RATIOS OVER TIME.

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ABSTRACT

Lipid subclass ratio has been shown to reflect deterioration of storage lipids in colonies of *Montastrea annularis* and *M. faveolata* subject to sedimentation stress. Lipid subclass ratio may be a viable tool in evaluating non-lethal changes in coral condition over short time scales but traditional methods of tissue collection (removal of small colonies or large chunks of coral skeleton) are too destructive to warrant repetitive use. In this study the ratio of storage lipid (wax ester and triacylglyceride) to structural lipid (sterol ester, primarily cholesterol and phosphoplipid) was measured in colonies of *Montastrea annularis* subject to sedimentation stress to determine the minimum sample size required for analysis. The study was repeated and tissue was collected with a minimally destructive technique using Vacutainer® tubes to remove individual polyps without destruction or removal of skeleton. Colonies of *Montastrea annularis* received a single, experimental dose (< 5.0 mg/cm^2) of locally obtained sediments. Lipid ratio was consistent to for 5 polyp samples but showed increased variability as sample sizes decreased below three polyps. Lipid ratio fell predictably (mean 0.9 to 0.36, n = 7-17) with treatment for samples collected using the Vacutainer® tubes ($p < 0.001$). This study indicates that the lipid ratio is consistent over extremely small sample sizes and may provide a tool to monitor and assess changes in coral nutritional condition during periods of environmental stress.

INTRODUCTION

In their summaries of the effects of sedimentation effects on hard coral Rogers (1990) and Brown and Howard (1985) address the tremendous range, and contradictory outcomes of coral response to sediment stress. Brown and Howard (1985) provide numerous examples of resistance to, and recovery from, severe sedimentation in both the field and laboratory while other studies indicate that small changes in sediment regime result in the destruction of healthy reefs (Loya, 1976, Hands et al., 1993, van Katwijk et al., 1993).

This apparent "paradox" of effects may result from a failure to adequately reflect matters of scale when considering changes in reef community structure (Harriott, 1994). Most experimental investigations are performed on the individual colony, organism-level over relatively short time scales (hours to weeks), while most analysis of coral reef community change (percent live cover and species diversity) can only be identified over relatively long time scales (years to decades). Peters and Pilson (1985) suggest a comprehensive protocol for evaluating coral community response to environmental stress through the long-term monitoring of random individuals. The list includes 1. Visual observations (polyp condition, lesions, mucus, bleaching, presence of sediments); 2. Histological examinations (reproductive cycle and development, morphological changes, presence of microparasites or pathogens) and; 3. Physiological measurements (metabolic rate, photosynthesis rate, calcification and growth rates, biochemical composition) of individual colonies to determine how individual-level change is manifest at the community level. While long-term visual observations and monitoring have continued to

provide valuable information there has been relatively little biochemical and other cellular-level research designed to quantify sub-lethal responses to environmental stress. While Somero (1992) argues that biochemical adaptation and response determine ecological patterning in marine systems, little research has been done to identify how cellular level response is manifest in hard coral community structure.

Harriott (1994) reinforces Peters and Pilson's (1985) need to evaluate physiological and histological condition. Harriott (1994) argues that the inconsistencies in response to stress from the organism to the community level clearly suggest a need to develop indicators of coral condition that can link change at the organism level with changes at the community level. Rogers (1990) too, echoes the suggestions of Peters and Pilson (1985) but cautions that physiological and histological investigations often only provide information about organisms that have survived the environmental stress. Clearly, while biochemical indices may provide the tools to evaluate short-term changes in coral condition which are also relevant to long-term changes in condition at the individual and community levels, any index must permit the repeated sampling of individuals over time (i.e. be relatively non-destructive).

Changes in cellular protein, lipid and enzyme composition and activity have been evaluated for temperature (Dietz and Somero, 1992), salinity (Kueltz and Somero, 1996) and pressure (Gibbs and Somero, 1990, Somero, 1992) in marine vertebrates and invertebrates and the relative amounts of storage lipid to structural lipid (WE+TAG):(SE+PL) have been used as a condition index in other organisms (Fraser,

1983, Emmett et al., 1987, Adams et al., 1988, Pollero et al., 1991, Håkanson, 1993). Furthermore, the ratio of lipid subclass has been shown to reflect stress response in *Montastrea annularis* and *M. faveolata* (Chapters 2 & 3).

The use of a lipid ratio provides a dimensionless index of coral condition and, lipid ratio, as a relative index of lipid quality, eliminates dependence upon tissue sample weight and reduces the sampling difficulty of measurement.

Lipids are important in coral energy storage and reproduction (Ducklow and Mitchell, 1979) and, like other organisms in nutrient limited environments, corals must balance the nutritional expense of reproduction against the nutritional demands of growth, development and normal metabolism (Ward, 1995). Excess nutrient use can deplete nutrient reserves and depress, or eliminate, reproductive activity, growth or development. The detrimental affects of inadequate lipid reserves are manifest on the organism level during the short-term (decreased tissue lipid levels, decreased colony reproductive potential and decreased colony growth) but will also be evident in the community level, over long-time scales, as changes in species diversity and percent live cover (Tomascik and Sander, 1987, Ward, 1995).

Coral nutrient reserves are stored as wax esters (WE) and triacylglycerides (TAG)(Patton et al., 1977). Levels of WE and TAG can fluctuate with coral reproductive state (Stimpson, 1987), food availability, metabolic requirements (Crossland et al., 1980, Crossland, 1987) and productivity of algal symbionts (Patton et al., 1977, Stimpson,

1987). The polar lipids (including the phospholipids, PL) and the sterol esters (SE, primarily cholesterol) are structural lipids and remain relatively stable with short-term changes in nutritional state (Meyers, 1979). Free Fatty Acids (FFA) are rarely present (Harland et al., 1993, Chapter 2) and large quantities of FFA represent lipid catabolism (Chapter 3).

Coral lipid subclass ratio has been shown to reflect the increased use of stored lipids in colonies of *Montastrea annularis* and *M. faveolata* subject to sedimentation stress under experimental and natural conditions. Lipid subclass has also been shown to reflect lipid catabolism in heat-stressed colonies of *Montastrea annularis*. Lipid ratio, therefore, is a useful tool in evaluating individual-level response to stress and the repeated monitoring of coral colonies may provide insights in to the linkage between short-term, colony effects of sedimentation (death, bleaching, reduced P:R, recovery) and the long-term community effects of sedimentation (reduced colony growth, decreased species diversity, decrease in percent live cover). The standard procedure of removing large samples of coral (hammer and chisel) from the subject colonies is too destructive to use with regularity in a given study site and any monitoring protocol for coral must be largely non-destructive if it is to be used with regularity, repeatedly or in large numbers.

This study examines changes in lipid subclass extremely small tissue samples of the coral *Montastrea annularis* subject to sedimentation stress and evaluates the potential use of lipid subclass as a minimally destructive method of determining nutritional state in coral.

METHODS AND MATERIALS

Lipid ratio and sample size

Ten colonies of *Montastrea annularis* were randomly selected from small, leeward patch reefs adjacent to the northwest coast of Vieques, Puerto Rico (18°06'34" N, 65°34'32"W)(Figure 9) and marked for use in the sedimentation experiments from July 14-19, 1997. Tissue samples for this study represent a sub-set of the control and experimental samples presented in Chapter 2.

Five replicate tissue samples were subdivided into a five polyp sample, a three polyp sample and a one polyp sample for evaluation of lipid ratio over decreasing tissue sample size. Coral tissue was removed from the carbonate skeleton of control colonies and experimental colonies, manually, using fine forceps, placed in pre-weighed amber collection vials and freeze-dried using a VIGRIS (Gardiner, NY) desiccator/freezer. The vials containing the dried tissue were re-weighed to obtain a dry weight and frozen until processing.

Micro-tissue collection

Ten colonies of *Montastrea annularis* were randomly selected from small, leeward patch reefs adjacent to the northwest coast of Vieques, Puerto Rico (18°06'34" N, 65°34'32"W) and marked for use in the sedimentation experiment from April 7-8, 1998. (Figure 9). Five, replicate, pre-treatment samples were removed from each control and experimental coral using micro-tissue collection techniques described below. Locally

obtained sediments were collected, dried and weighed to determine an approximate, standard weight/area of 5.0 mg/cm^2 for each dose using a pre-marked scoop. Sediment was applied to the 5 experimental colonies after Rogers (1983). Five control colonies did not receive any applied sediment. After 24 hours, post-treatment samples were removed as above.

Coral tissue was removed from sample colonies using a Vacutainer® blood collection tube (Becton Dickinson, 10 ml draw) with a 20 x 2.0 mm gauge Vacutainer® blood collection needle. The needle was inserted into an individual coral polyp and rotated around the interior of the calyx to free the coral polyp tissue before collection. After the tissue was loosened, the Vacutainer® tube was placed on the needle, creating suction and transferring the tissue into the collection tube. The needle was then removed until the next use. This procedure was repeated to collect 5 polyps in each tube. Needles were exchanged between colonies to avoid cross contamination of the tissue. The collection tubes containing the tissue samples were placed in a cooler and returned to the laboratory. Tissue samples were transferred to amber vials containing 1.0 ml methanol:chloroform (2:1 v/v) using a Pasteur pipette and frozen for return and qualitative analysis at the Virginia Institute of Marine Science.

Lipid analysis

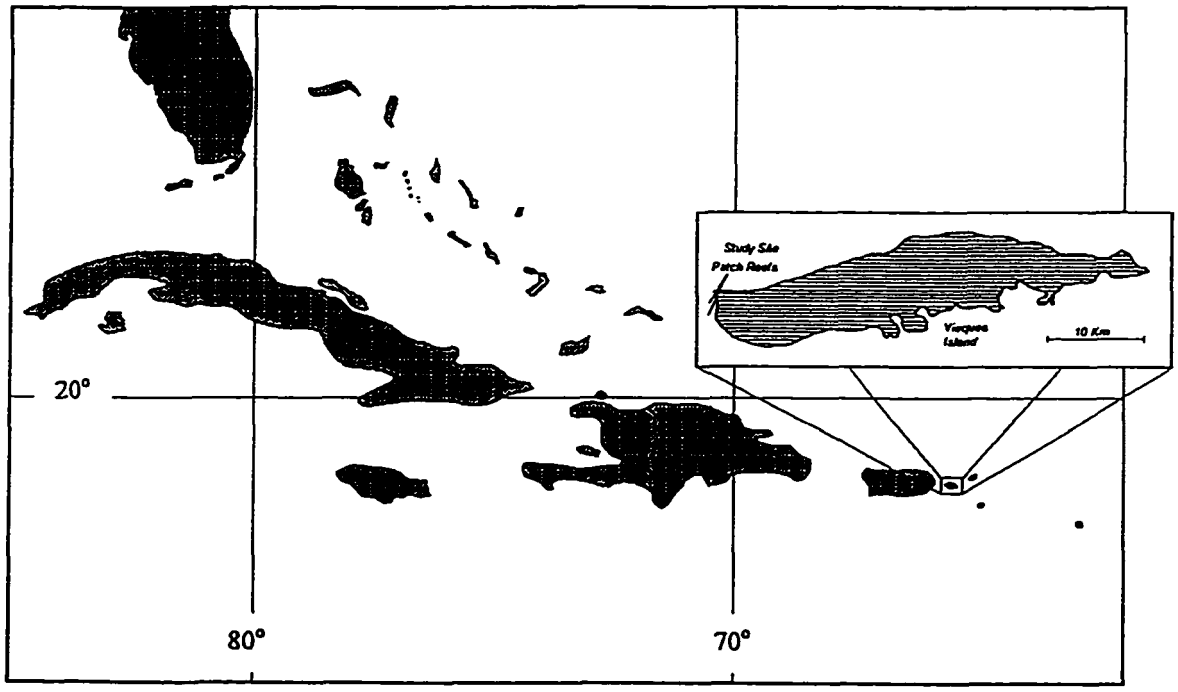
Lipid class content was determined using an Iatroscan thin layer chromatograph (TLC) with a flame ionization detection (FID) system (Iatroscan Laboratories Inc., Tokyo Japan). Coral tissue lipids were extracted twice from homogenized polyp tissues using

modified methods of Folch et al. (1957) and Bligh and Dyer (1959). Briefly, this procedure separates and extracts lipids from tissue in a biphasic solution of chloroform/methanol/ water (2:2:1, v/v). Approximately 10-25 mg of dry sample was extracted for lipid class analysis. The lipid phase was captured, pooled for the two extractions, and evaporated to dryness to concentrate the sample to 100 µl. Two (2) µl of concentrated lipid extract were spotted on S-III chromarods and lipid classes were developed in hexane:diethyl ether:water (85:15:0.04) to separate the lipid classes. The chromarods were scanned and lipid classes were determined by standard curve on using a Mark III Iatroscan analyzer.

Data analysis

Statistical comparisons of experimental results are, unless otherwise noted single factor ANOVAs. The use of individual tissue samples taken from the same colony as independent samples has been widely used (Meyers et al. 1978, Meyers, 1979, Harland et al, 1992, Kinzie, 1993, Latyshev et al., 1991) and was used in this study when paired tests were impossible or inappropriate (lost or destroyed "pairs"). Unless otherwise noted, coral tissue lipid responses to sedimentation were measured and presented as the ratio of storage lipid to structural lipid and statistical tests were made using arcsine transformed proportions.

Figure 9. Location of the study site at Vieques, Puerto Rico.



RESULTS

Total lipid was variable with decreasing sample size (Figure 10) ranging from mean levels of 57.9 mg lipid/g dry tissue (n = 5, s.e. 13.3) in the 2 polyp, experimental sample to a level of 8.06 mg lipid/g dry tissue (n = 5, s.e. 2.82) in the 1 polyp experimental sample. Variability was not as large in the control samples with a range of 10.95 – 29.38 mg lipid/g dry tissue (n = 5, s.e. 5.13 – 8.60). Lipid ratio was not statistically different with decreasing sample size in either experimental colony (ANOVA, $p > 0.30$) or control colony ($p > 0.25$) samples.

Micro-tissue collection and sedimentation

Experimental colonies of *Montastrea annularis* experienced a one-time episode of sediment loading equal to approximately 5.0 mg/(cm^2). Background sedimentation rates were 0.0 mg/(cm^2 day) for this study. The ratio of (WE+TAG): (SE+PL) in coral colonies exposed to sedimentation (0.31, s.e. = 0.04) differed significantly from pre-test (1.04, s.e. = 0.12) and post-test control colonies (1.1, s.e. = 0.27) and from pre-treatment levels (0.91, s.e. = 0.12) in experimental colonies (ANOVA, $p < 0.001$)(Figure 11).

Figure 10. Mean levels (± standard error) of *M. annularis* coral tissue lipids compared to sample size. A. Mean (n = 5) level of Total Lipid (mg lipid/g dry tissue weight) for post-treatment control (●) and experimental (▲) coral tissue with decreasing sample size (from the same colony). B. Mean (n = 5) ratio of storage to structural lipids (WE+TAG:SE+PL) for control (and experimental coral tissue with decreasing sample size (from the same colony).

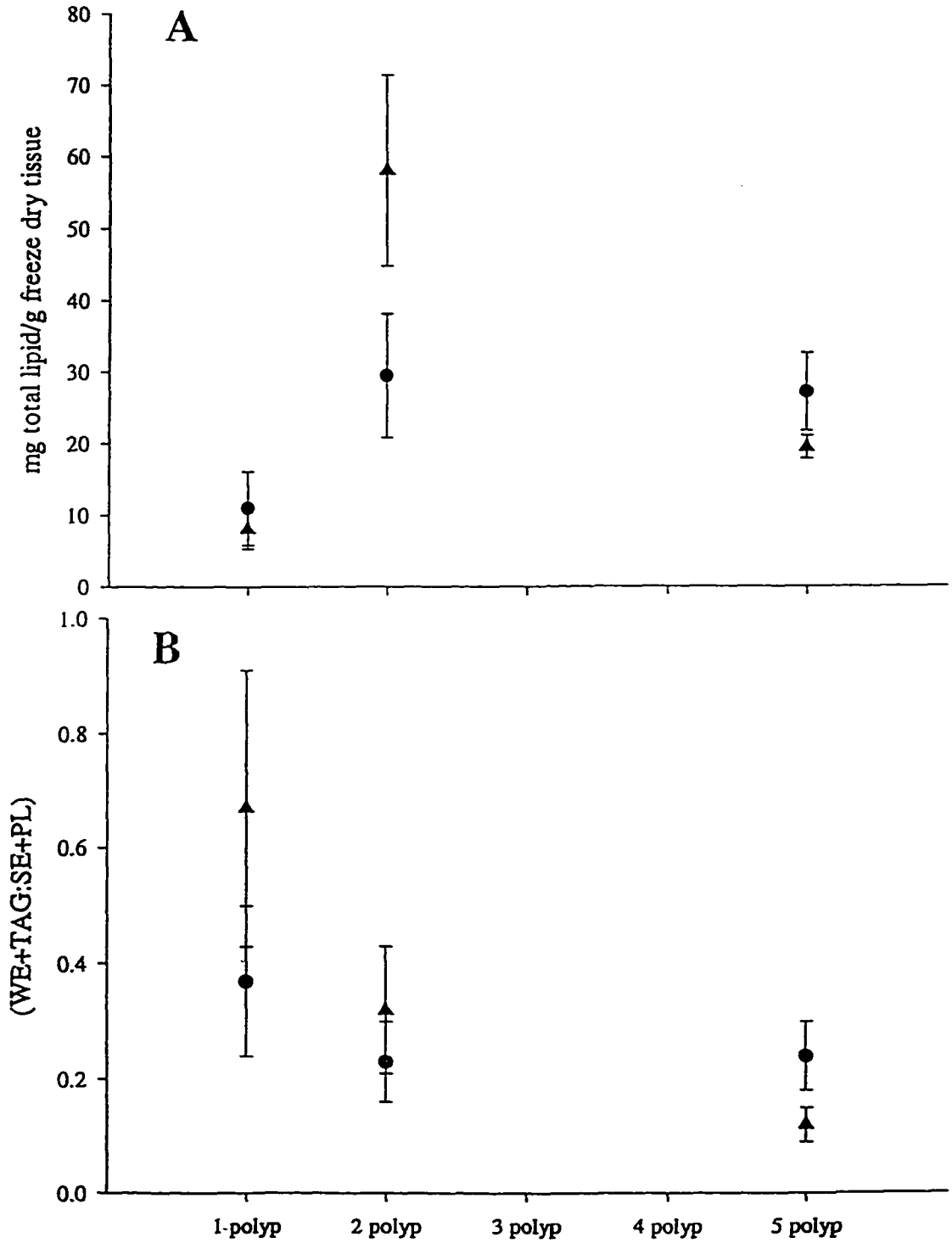
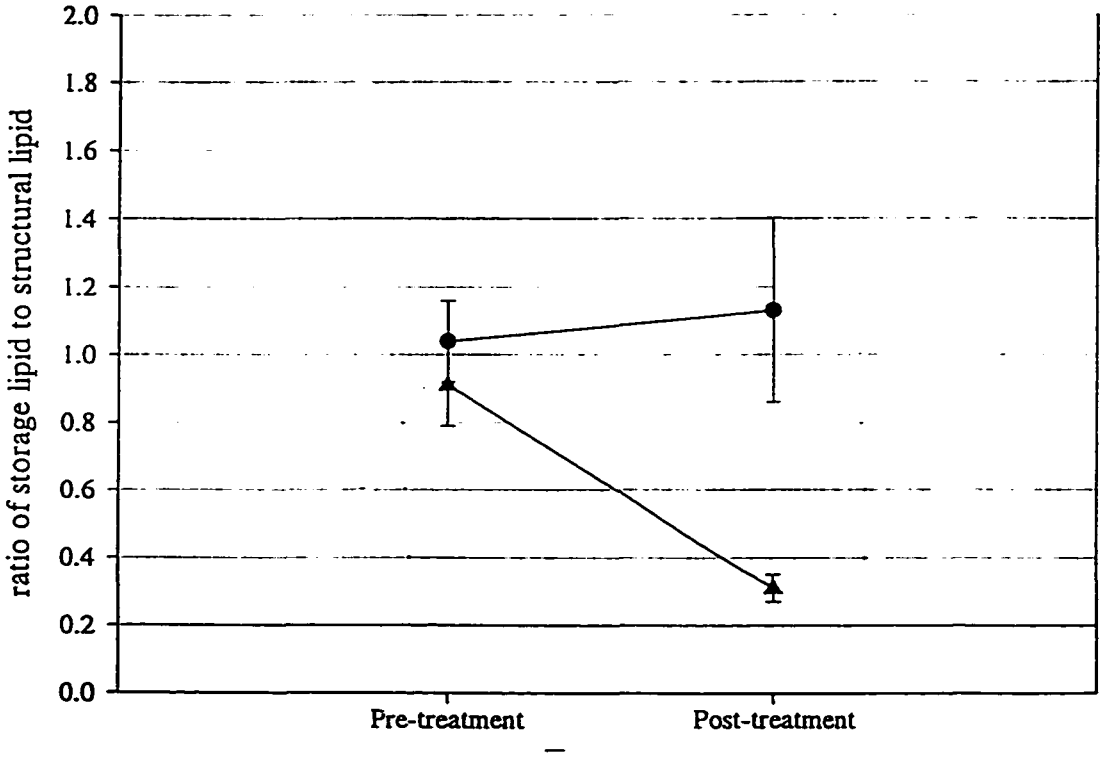


Figure 11. Mean ratio of storage to structural lipid in colonies of *M. annularis* subject to sedimentation stress using tissue samples (5 polyp) collected without removal of coral skeletal tissue. Error bars represent standard error. Levels of (WE+TAG):(SE+PL) dropped significantly with treatment ($n = 7\text{-}17$, ANOVA $p < 0.001$, Tukey Means Comparison Test $p < 0.001$).



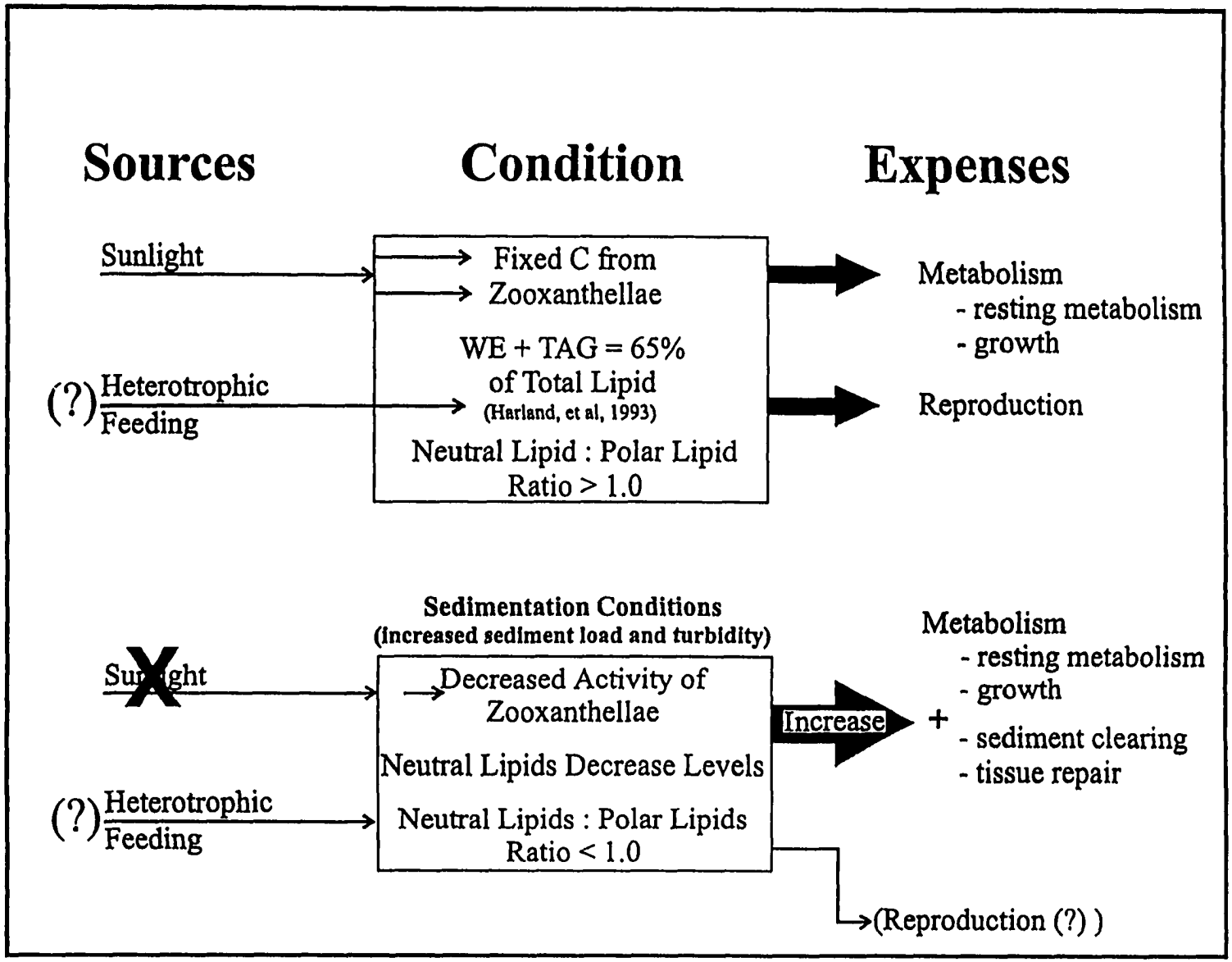
DISCUSSION

The results of this study indicate that extremely small tissue samples can be used to determine changes in the ratio of storage lipids to structural lipids in colonies of *M. annularis* subject to sedimentation stress. While a theoretical argument could be made to support collection of as few as two polyps based upon statistics (Figure 10), the mechanics of handling small tissue samples require the larger sample size to reduce the number of samples lost in transfer from the collection tube to the extraction solution. Corals subject to experimental sedimentation stress exhibited the predicted decrease in the ratio of storage lipid to structural lipid and the ratio of storage to structural lipids (WE+TAG:SE+PL) in sediment-stressed colonies of *M. annularis* fell significantly compared to control colonies and pre-treatment levels despite the use of Vacutainer tubes for minimally destructive collection (Figure 11).

Lipid ratio has been previously suggested to be an indicator of short-term sedimentation stress in *Montastrea annularis* (Chapter 2). The decrease in lipid ratio may result from decreased net production of zooxanthellae (Stimpson, 1987, Telesnicki and Goldberg, 1993), and/or increased metabolic demand associated with sediment clearing (Abdel-Salam et al., 1988), including loss of lipid in mucus involved in sediment clearing (Stafford-Smith and Ormond, 1992, Stafford-Smith, 1993).

In addition to providing energy for reproduction (Battey et al., 1998) excess lipid reserves can prevent (or delay) the expulsion of zooxanthellae by maintaining adequate nutrient levels under environmental conditions which would typically stress the symbiotic relationship (Patton et al., 1977, Crossland et al., 1984, Crossland, 1987, Stimpson, 1987). Under ideal environmental conditions, symbiotic corals maintain the metabolic demands of growth and tissue repair while accumulating excess energy in the form of lipids (primarily WE and TAG)(Stimpson, 1987, Harland et al., 1993). A nutritional state diagram for *Montastrea annularis* is presented in Figure 12, and describes nutrient sources and expenses for stressed and non-stressed conditions. The paradox between variable coral response to sedimentation stress at the individual level and the manifestation of those responses on the community level (Brown and Howard, 1985, Roger, 1990) may be explained by variability in colony growth, reproductive success and survival under sub-optimal conditions. Ratio of storage to structural lipids indicates the nutritional status of coral over short time scales and provides insight into Somero's (1992) judgement that ecological condition in marine systems is determined by biochemical adaptation. Lipid ratio also reflects short-term coral response to environmental degradation and may address Harriott's (1994) call to develop indicators of coral condition that can link change at the organism level with changes at the community level. Evaluated over time, lipid ratio may provide a biochemical link between colony level stress and reef community change and may shed insight into the variability of coral colony, and reef level, response to minor environmental stress.

Figure 12. A simple nutritional state diagram for hard coral under non-stressed and sediment stressed conditions.



Lipid ratio has been shown to reflect coral nutritional condition under sedimentation stress, despite the collection of small sample sizes. The use of Vacutainer tubes provides a minimally, destructive sampling technique the does not cause structural damage to the colony. This procedure may allow repeated monitoring of lipid ratio in large colonies. Repeated biochemical monitoring of coral condition, over time, may provide insight to many cellular level and physiological conditions. Future study may include cellular level response to environmental stress, determination of physiological changes that trigger sexual reproduction and the relative contributions of zooxanthellae and heterotrophic feeding. Long-term monitoring may also provide insight to how individual coral response is manifest on the community level.

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CHAPTER V

CORAL LIPID AND ENVIRONMENTAL STRESS: SUMMARY OF FINDINGS AND OPPORTUNITIES FOR FUTURE RESEARCH

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CHAPTER V

CORAL LIPID AND ENVIRONMENTAL STRESS: SUMMARY OF FINDINGS AND OPPORTUNITIES FOR FUTURE RESEARCH

It is generally accepted that, on a global basis, coral reefs are in a state of decline due to increased natural and anthropogenic stress. There is disagreement to the level of this decline and the mechanisms for accurately evaluating the causal relationship of these detrimental effects have not yet been identified. Harriot (1994) argues that traditional, visual, benthic sampling of hard, coral reef cover is not adequate to determine subtle changes in the reef environment and does not identify the source or effect of small changes in environmental condition. Peters and Pilson (1985) suggest that additional measures of physiological effects of environmental stress must be found in order to overcome the inadequacies of traditional visual monitoring. Such measures would be able to link small changes in the physical environment with changes in hard coral community structure. The lack of a suitable physiological index of coral condition has hindered the examination of the linkage between short-term environmental stress and the manifestation of individual responses on the community level. Stimpson (1987) encouraged the use of lipid as a condition index in coral based upon the observed decrease of total lipid after an experimental decrease in available light and after planulation. The use of lipid as a condition index seemed appropriate because of the large levels of lipid found in coral tissues and the role of lipid in coral energy storage, growth,

and reproduction. Accurate determination of total lipid, however, proved to be difficult and subject to large sampling errors (Harriott, 1994).

Total Lipid, Lipid Ratio and Environmental Stress

The present study appears to indicate that the use of lipid class ratios provides accurate, qualitative data on coral nutritional condition on short time scales. The use of lipid class ratio can also be measured repeatedly, on the same corals, over time to evaluate coral nutritional condition with changes in the physical environment.

Measurements of total lipid were highly variable in control and treatment organisms throughout these experiments. Variability was high in both pre-treatment and post-treatment samples and total lipid did not change significantly with treatment under sedimentation or heat stress conditions. These findings are coincident with those of Harriott (1994) and reinforce the difficulties associated with the use of total lipid as an index of coral condition. The unexpected presence of large levels of Free Fatty Acids (FFA) in the heat-stressed samples indicated catabolism (or oxidation) of lipids in the coral tissues and may reflect a decrease in the level of total lipid. However, possible decreases in the level of total lipid in post-treatment, heat-stressed tissues were not statistically identifiable due to the large variance of the data. The difficulty in measuring accurate total lipid levels is a primary deterrent to using total lipid as a stress indicator in coral (Harriott, 1994).

Although the total lipid level proved to be a poor indicator of condition, the ratio of lipid classes provided some insight into individual coral response to both minor and severe environmental stress. Under minor sedimentation stress, coral colonies of *Montastrea annularis* and of *M. faveolata* exhibited an immediate, significant decrease in the ratio of storage (wax esters and triacylglycerides) to structural (sterol esters and phospholipids) lipids. The decrease in this ratio indicates a reduction in stored energy in these corals. The use of stored energy has been suggested as a mechanism to maintain a stable cellular environment under conditions of moderate environmental stress (Patton et al., 1977, Crossland et al., 1980, Crossland, 1987, Stimpson, 1987). A stable cellular environment, proper balance of oxygen and nitrogen levels, is necessary to maintain the delicate balance of the host-zooxanthellae relationship (Crossland et al., 1980, Lesser et al., 1990, Lesser, 1997). The fatty acid composition of coral tissues subject to minor sedimentation stress reflected a reduction in the presence of the algal-derived polyunsaturated fatty acids (PUFA) in the polar lipids indicating a relative reduction in zooxanthellar membranes. The reduction in the number of zooxanthellae (or reduction in the number of chloroplasts in each zooxanthellae) in the cells may account for this reduction in the algal polar lipids. Reduction in number of zooxanthellae is consistent with maintaining this cytoplasmic stasis by decreasing the potential for oxygen production (Crossland et al., 1980, Lesser et al., 1990, Lesser, 1997, Ferrier-Pagét et al., 1998, Jones et al., 1998). The ratio of storage to structural lipid recovered quickly to pre-sedimentation levels in *M. faveolata* located in the undisturbed, Lee Stocking Island, Bahamas study site.

Under severe heat stress conditions however, the above maintenance strategy was not evident. *Montastrea annularis* subject to severe heat stress exhibited a dramatic increase in tissue Free Fatty Acids (FFA) while the ratio of storage to structural lipids did not change. The increase in FFA indicated the catabolism (or oxidation) of tissue lipids (Sheridan, 1988, Fang et al., 1997). Analysis of the fatty acid composition of these tissue samples indicated that the majority of the change occurred in the polar lipid fraction PUFA. This reduction of polar lipid PUFA (and the associated, concomitant increase in the relative amount of saturated fatty acids) may result from "stiffening" (replacing the heat-pliant PUFA with more rigid, saturated fatty acids) of the membrane under high temperature conditions but also indicates a significant loss of algal membranes. The increase in FFA along with the loss of algal membrane lipid supports previous studies that suggest the preferential digestion of zooxanthellae as one defense mechanism under severe, heat-stress conditions (Hoegh-Guldberg and Smith, 1989, Szmant and Gassman, 1990, Suharsono et al., 1993).

The different lipid subclass ratios appear to indicate two possible cellular-level mechanisms in response to environmental stress. The first mechanism may be a *maintenance strategy* that uses stored lipid to maintain the symbiosis during periods of minor, short-lived stress. The second appears to be a *defensive strategy* involving the catabolism of tissue lipids (including the preferential loss of algal membrane lipid) to remove zooxanthellae (and associated oxidative stress during heat stressed conditions) as quickly as possible from the host tissues (Hoegh-Guldberg and Smith, 1989, Szmant and Gassman, 1990, Suharsono et al., 1993).

Evaluating and Monitoring Coral Tissue Lipid

The principal deterrent to examining coral physiology through time and under a variety of conditions has been the lack of a technique that does not result in the destruction of the sample colony. The use of Vacutainer® blood collection tubes to collect samples, rather than the traditional methods of tissue collection which remove large chunks of coral skeleton and tissue using hammer and chisel, enables researchers (and resource managers) to perform repeated sampling of the same coral head. Although lipid ratio can be accurately assessed using sample sizes as small as two polyps (30-50 μg) using the Vacutainer technique, a sensitivity analysis indicates the use of a larger number of polyps (3-5 polyps) is prudent.

Limitations of the Present Study and Opportunities for Future Research

The present study indicates the use of lipid class ratio as an indicator of both minor and severe environmental stress, yet a lack of baseline information regarding normal fluctuations, or differences, in the amounts of each lipid class in hard coral can not be overlooked. Basic information regarding both the quantity and quality of lipids in coral has rarely been studied and little conclusive data is available for comparison between "healthy" and "stressed" corals (Stimpson, 1987, Latyshev et al., 1991, Harland et al., 1992). Even less information is available regarding the basic, isolated lipid biochemistry of either the host or zooxanthellae tissues, through time or over changes in reproductive state or environmental variation.

To address the inadequacies of the present study (two species, different study sites and only two experimental conditions) and to broaden the underlying knowledge of lipid biochemistry in hard coral I suggest the following future investigations:

To Evaluate Basic Lipid Composition

- Identify the lipid class and fatty acid compositions of isolated zooxanthellae (for each possible species and clade) found in *Montastrea annularis* and *M. faveolata.*

- Identify the lipid class and fatty acid compositions of coral host tissue in the absence of zooxanthellae (bleached tissues, starved and fed) in *Montastrea annularis* and *M. faveolata.*

- Further identify the typical lipid class and fatty acid compositions of combined host and zooxanthellae tissues for other species of coral.

To Evaluate Nutritional Evidence of Change Resulting From Stress

- Investigate changes in lipid class and fatty acid compositions in the same species (and morphotype) from the same location at several different depths to determine possible effects of decreasing light on coral nutritional state and to identify possible differences in the relative contribution of zooxanthellar production versus feeding.

- Experimentally investigate changes in lipid class and fatty acid compositions in the same species (and morphotype) from the same location at several different temperatures to determine possible effects of heat on coral nutritional state and to identify possible differences in the relative contribution of zooxanthellar production versus feeding.

- Conduct repeated sampling of the same coral colonies under non-stressed conditions to identify natural changes in lipid class composition over time to determine the frequency and amplitude fluctuations of lipid ratio through seasonal changes in the environment and the possible relationships between lipid class ratios and changes in reproductive state.

- Conduct repeated, long-term experimentation and evaluation of the effects of repeated minor sediment stress on lipid class ratio on the individual colony level to assess the range of short- and long-term response on the individual and community levels.

- Conduct repeated, long-term experimentation and evaluation of the effects of repeated minor heat stress on lipid class ratio on the individual colony level to assess the range of short- and long-term response on the individual and community levels.

These experiments should be repeated for other species of hard coral to evaluate the use of lipid class ratio as a broad range condition index for coral communities. The use of lipid class ratio has been shown to fluctuate predictably with the application of both minor and severe environmental stress. Long–term studies of the response of individuals to repeated stresses may provide a physiological link between individual, short-term responses to environmental stress and the long-term changes in reef ecology which become evident through long-term visual monitoring and may help to rectify the apparent paradox of responses which have been previously reported in the literature (Brown and Howard, 1985, Rogers, 1990).

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VITA

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Born in Long Beach, New York, 2 June 1961 and graduated from West Islip High School with highest honors in 1978. Earned an A.B in Biology and Pyschology from The College of William and Mary in Virginia in 1982. Completed his certification as a school teacher in 1989 and received his A.M. from The School of Marine Science of the The College of William and Mary in 1993. Entered the doctoral program at the School of Marine Science in 1993. Presently the education coordinator of the Chesapeake Bay National Estuarine Research Reserve in Virginia (CBNERR) and a senior marine scientist at the Virginia Institute of Marine Science (VIMS), he is responsible for the development of education programs for a variety of audiences from school children to lawyers and graduate students. He has spent time working as a commercial fisherman, a hospital administrator in public relations and marketing and as a teacher before becoming an environmental scientist and educator. He is also a blue-water sailor and he maintains a viable career as an entertainer/performer where he has been in several movies and television productions that feature historical music.

IMAGE EVALUATION
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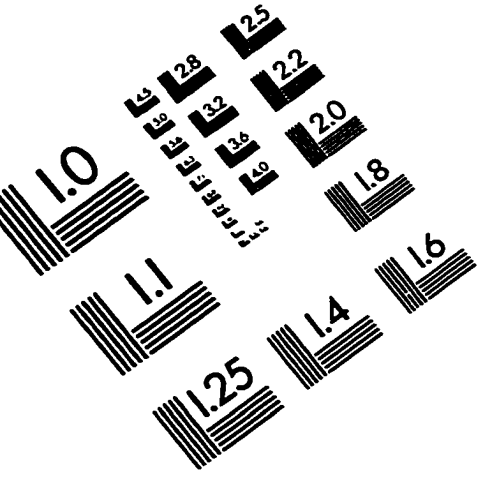

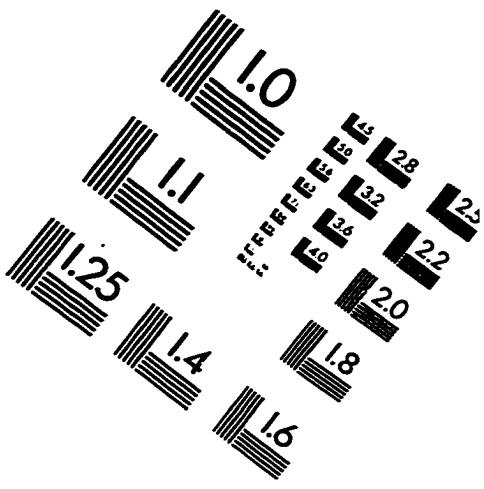

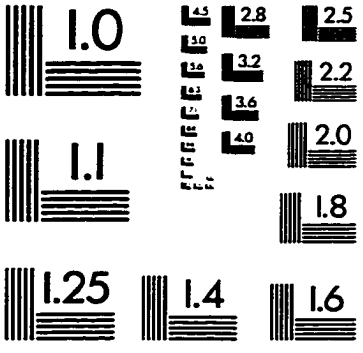

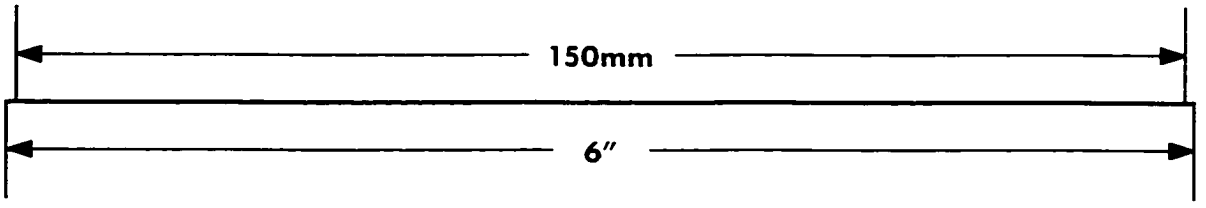

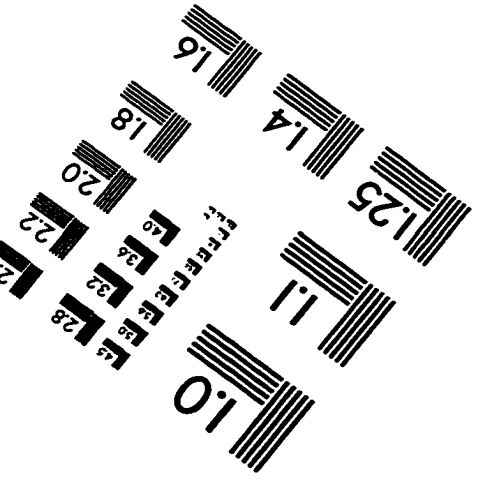

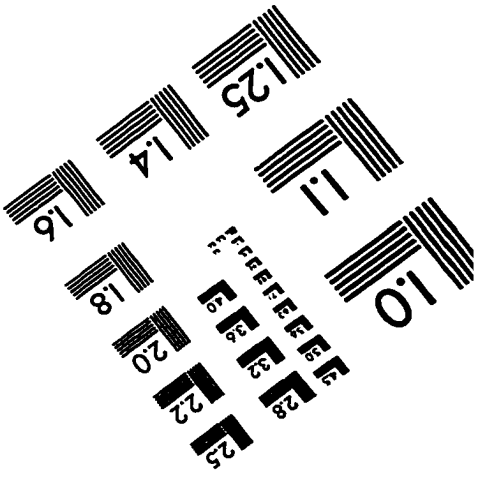

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