SPONGES IMPACTS ON CORAL REEF NITROGEN CYCLING, KEY LARGO, FLORIDA

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ABSTRACT

Melissa W. Southwell: Sponge impacts on coral reef nitrogen cycling, Key Largo, Florida
(Under the direction of Christopher S. Martens)

Sponges are potentially important drivers of nitrogen cycling on Caribbean coral reefs due to their capacity to filter large volumes of water, their dense microbial communities, and their large population size. Given the potential role that nitrogen plays in controlling primary production and reef health, it is important to understand and quantify the nitrogen fluxes between sponges, their associated microbial communities, and the surrounding water column. The main goal of this dissertation is to characterize the role of sponges and their microbial assiciates in reef N cycling, specifically: 1) to quantify the flux of dissolved inorganic nitrogen (DIN) to the water column, 2) to investigate the process of nitrification, and 3) to measure nitrogen fixation rates in sponges.

Nitrogen fixation rates in sponges were found to be very low relative to the ambient water column; therefore, sponge-hosted nitrogen fixation probably does not contribute significantly to sponge nutrition or to inputs of new nitrogen for the reef. However, sponges were found to be a large source of DIN. The DIN flux from the sponge community was measured using a combination of incubation experiments and a novel *in situ* method and found to be 660 ± 130 μmol m^{-2} h^{-1}, which is approximately ten times higher than other reported benthic nutrient fluxes. Most of this DIN is released in the form of nitrate due to active communities of ammonia oxidizers and nitrifiers hosted by many of the most abundant sponge species. At present it is unknown whether the ammonia-oxidizing community is dominated by archaea or bacteria, but the results of this study show that the isotopic fractionation and sensitivity to chemical inhibition are consistent with bacterial ammonia oxidizers. Because nitrogen fixation rates were negligible compared to DIN flux rates, sponge-

produced DIN is likely derived from the remineralization of organic matter. Therefore, although sponges do not appear to facilitate fluxes of new nitrogen for the reef, they do appear to regenerate large quantities of inorganic nutrients, and to facilitate the oxidation of ammonium to nitrate.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 2

Chapter 3

Appendix G

LIST OF SYMBOLS AND ABBREVIATIONS

AOA: Ammonia oxidizing archaea

Chl-a: Chlorophyll-a

$\delta^{13}C$: Expression of the isotopic composition of carbon in a sample, relative to the standard Pee Dee Belemnite. It is calculated according to the following:

$$\partial^{13}C = \left(\frac{R_{sample} - R_{standard}}{R_{standard}} \right) \times 1000, \text{ where } R = \frac{^{13}C}{^{12}C}$$

$\delta^{15}N$: Expression of the isotopic composition of nitrogen, analogous to the above equation, but where $R = \frac{^{15}N}{^{14}N}$

‰: Units in which δ values are expressed

ΔDIN: Concentration of DIN in sponge excurrent water (water expelled by the sponge) minus ambient water adjacent to the sponge.

DIN: Dissolved inorganic nitrogen, including NH_4^+, NO_2^-, and NO_3^-

DOM: Dissolved organic matter (DON = dissolved organic nitrogen)

LMA: Low microbial abundance (*sensu* Hentschel et al. 2006), referring to species of sponge with microbial communities of similar composition and density to the water column

HMA: High microbial abundance, referring to species of sponges containing large internal microbial communities.

POM: Particulate organic matter

SPOM: Suspended particulate organic matter

CHAPTER 1

Sponges in a changing ecosystem

Although the coral reef ecosystem is composed of a highly diverse, interconnected group of organisms, a cursory survey of the literature reveals that studies on coral reef health focus disproportionately on the coral animals themselves. This is not surprising because corals are a critical, yet fragile, component of the ecosystem: they generate the hard structure that provides refuge and substrate for many other organisms. Furthermore, phenomena such as coral bleaching, disease, and predation by Acanthaster have increased interest in understanding how these animals function and cope with environmental stress. However, while acknowledging the central role of corals, it is important to consider that other organisms on the reef also have essential functions in reef ecology.

Sponges, for example, are abundant on many reefs, and in some Caribbean regions, they are the most abundant of all taxa (Diaz and Rutzler 2001). Sponges are known to perform several ecosystem services, such as stabilizing substrate (Wulff and Buss 1979) and providing habitat for small invertebrates (Butler et al. 1995). They may also play a large role in recycling of small particulate organic matter (POM) because of their ability to filter large volumes of water on a daily basis. The the metabolic waste released from this activity can have a significant effect on POM and nutrient concentrations in the water column. In the coral reef ecosystem, recycling of nutrients is a critical function that allows this system to

maintain high primary productivity rates despite low nutrient concentrations. However, elevated nutrient concentrations are thought to have deleterious effects on some reef organisms. Therefore, the potential for sponges to act as a source or sink for bioavailable nitrogen could be important for the health of other reef organisms, as well as the overall ecological balance of this system. This chapter focuses on the role of nutrients in coral reef decline, and the potential for sponges to affect nutrient availability. Although much of the literature reviewed in this chapter has an unavoidable coral bias, it is important to remember that environmental factors affecting coral health may also impact other important reef organisms. Furthermore, organisms such as sponges may not simply be the passive recipients of external forces causing environmental change; rather, they may also exert positive or negative feedbacks that accelerate or mitigate such external forces.

Coral reef decline

> The apparently capricious distribution, therefore, of coral-reefs, cannot be explained by any … obvious causes; but as the study of the terrestrial and better known half of the world must convince every one that no station capable of supporting life is lost,--nay more, that there is a struggle for each station, between the different orders of nature,--we may conclude that in those parts of the intertropical sea, in which there are no coral-reefs, *there are other organic bodies supplying the place of the reef-building polypifers*. …The relations, therefore, which determine the formation of reefs on any shore, by the vigorous growth of the efficient kinds of coral, must be very complex, and with our imperfect knowledge quite inexplicable. From these considerations, we may infer that changes in the condition of the sea, not obvious to our senses, might destroy all the coral-reefs in one area, and cause them to appear in another: thus, the Pacific or Indian Ocean might become as barren of coral-reefs as the Atlantic now is, without our being able to assign any adequate cause for such a change (Darwin 1842, emphasis by Southwell).

The above passage was written by Charles Darwin over 150 years ago, yet it is strangely prescient regarding the problems coral reefs are facing today. Since the time of Darwin, a considerable amount of energy has been devoted to understanding the nature and function of coral reefs. Yet despite this effort, the scientific community has been unable to predict or counteract their dramatic decline in the modern era, which has accelerated in the last several decades (Porter et al. 2002). Coral cover on reefs has declined by 30-80% worldwide, such that even reefs once considered nearly pristine (e.g., Great Barrier Reef) have now experienced significant degradation (Pandolfi et al. 2003). Caribbean reefs have suffered the most severe degradation, with an 80% reduction in live coral cover over 30 years (Gardner et al. 2003).

The reason for this decline is fiercely debated in the literature (Done 1992, Jompa and McCook 2002, Pandolfi et al. 2003, Bellwood et al. 2004, Lapointe et al. 2004, Littler et al. 2006a), but is generally ascribed to a combination of factors acting on local, regional, and global scales (Goreau 1992). These factors (in no specific order) include climate change (Glynn 1991, Brown 1997, Scavia 2002), increased disease occurrence and severity (Porter et al. 2001, 2002, Lafferty 2004), overfishing/reductions in herbivory (Hughes 1994, Williams and Polunin 2001), and increased nutrient levels (Lapointe 1997, 2004, Schaffelke and Klumpp 1998). Factors such as tourism pressure (Hawkins et al 1999), ship groundings, and blast fishing (Riegel 2001) can also be important on local scales. Natural phenomena (e.g., hurricanes) can also cause significant damage to reef structure as well as organisms (Gardner et al. 2005).

Low nutrient concentrations are characteristic of coral reef waters, and yet this ecosystem supports rapid rates of photosynthesis (Webb et al. 1975a), a paradox that has long

generated scientific interest in the metabolic pathways of reef organisms. High rates of N_2 fixation in algal turf, sediment, coral, and other substrates support much of this primary production (Johannes et al. 1972, Webb et al. 1975a, Wiebe 1975, Wilkinson et al. 1984, Williams et al. 1987, Capone et al. 1992, Shashar et al. 1994). Allochthonous sources of nutrients and organic matter can also contribute to the biogeochemical budget of the reef, including deep water upwelling (Rissik et al. 1997, Leichter et al. 2003), and terrestrial input, via runoff or groundwater seepage (Sammarco et al. 1999, D'Croz et al. 2005, Marion et al. 2006, Paytan et al. 2006). Although the metabolic systems that allow corals to flourish in oligotrophic waters have long been studied (Muscatine 1973, Muscatine and Porter 1977), the effect of increasing nutrient availability on these and other organisms is less well understood.

The ability of corals, not only to function, but also to successfully compete for space and light in a eutrophic system is highly controversial (Lapointe 1997, Hughes et al. 1999, Lapointe 1999, Aronson and Precht 2000, Szmant 2002, Lapointe et al. 2004). One reason for this controversy may be the difficulty in creating a manipulative experiment that accurately mimics processes occurring *in situ* over appropriate spatial and temporal scales (Littler et al. 2006b). Further, differences in macroalgal community composition and nutrient sources can impede the ability of researchers to generate an over-arching conclusion that can be applied to other regions. Thus, despite the wealth of literature dedicated to this subject, predicting the response of coral reefs to increases in nutrient availability remains a difficult task. Unfortunately, the land use changes and increases in agricultural loading associated with a growing global population are likely to further increase coastal eutrophication in the near future (Galloway et al. 2004). Therefore assessing the responses

and potential feedbacks of reef organisms to eutrophication is an important goal for marine scientists.

Symptoms of a degraded ecosystem

Eutrophication has been identified as a potential causative factor for several alarming phenomena in recent years. The increase in abundance of macroalgae on Caribbean coral reefs is one very visible change that has occurred (Done 1992, Hughes 1994, McManus and Polsenberg 2004). This type of ecological "phase shift" can be rapid and dramatic, and may or may not be reversible (Edmunds and Carpenter 2001, Idjadi et al. 2006). In some instances (e.g. Discovery Bay, Jamaica), algal overgrowth appears to be controlled by reductions in specific herbivores, such as the rapid decline of *Diadema antillarum* in the 1980's (Carpenter 1990, Hughes 1994, Aronson and Precht 2000). Studies from other regions generally support the hypothesis that herbivory strongly influences algal cover (Shulman and Roberson 1996, Williams and Polunin 2001, Diaz-Pullido and McCook 2003). However, many experimental and modeling studies show that elevated nutrient concentrations combine synergistically with reductions in herbivory and disturbances to intensify the effect and to impede the ability of the ecosystem to recover after a disturbance (Hughes and Connell 1999, McCook 1999, Smith et al. 2001, 2006, McManus and Polsenberg 2004, Jompa and McCook 2002, Littler et al. 2006, Mumby et al. 2006).

In a 2001 survey of coral disease incidence in the Florida Keys, a four-fold increase was found compared to 5 years prior (Porter et al. 2001). Although diseases are not a new phenomenon, the frequency and severity of the outbreaks have increased in recent decades (Aronson and Precht 2001, Porter et al. 2001), and the potential role of macro- and micro-

nutrients has been the focus of several studies (Lapointe 1997, Littler et al. 2006, Koop et al. 2001, Kline et al. 2006, Kuntz et al. 2005). Experimental evidence suggests that inorganic nutrients do not increase disease occurrence (Szmant 2002, Kuntz et al. 2005) but that dissolved organic matter (DOM) can facilitate coral diseases by increasing the metabolic rate of the microbes that live on the surface of the coral (Kline et al. 2006, Kuntz et al. 2005). However, inorganic nutrients may increase the production of DOM by stimulating primary production (Anderson and Zeutche 1970). Further, inorganic nutrients have been shown to increase the virulence of coral pathogens (Bruno et al. 2003) and to reduce coral fecundity (Koop et al. 2001). Therefore, nutrient loading likely promotes disease-related mortality, and may reduce further coral recruitment.

The role of sponges on coral reefs

Sponges are abundant on many reefs, and in the Caribbean they represent the dominant taxa (Diaz and Rutzler 2001). As filter feeders, a major food source for sponges is ultra- and picoplankton, and they are able to retain 58-99% of particles in the ultraplankton (< 5 μm) size fraction (Pile et al. 1996, 1997, Ribes et al. 2005). The majority of POM in reefs waters is in this size fraction (Ferrier-Pages and Gattuso 1998, Ferrier-Pages and Furla, 2001, Charpy 2005), and bacteria, in particular, appear to be an important food source for benthic organisms (Bak 1998, Gast, 1998, Ferrier-Pages and Furla 2001). The remineralization of this organic matter represents a significant source of nutrients (Hollibaugh 1991, van Duyl 2002), therefore organisms that facilitate this remineralization may represent and important sink for particulate N and source for dissolved inorganic N. By

preferentially grazing on small cells, sponges not only access a large reservoir of food, but also potentially impact the distribution of energy and nutrients among reef organisms. For example, the removal of pico- and ultraplankton coupled with the nutrient flux from their remineralization (Scheffers et al. 2004) could provide a competitive advantage to larger phytoplankton such as diatoms (van Duyl et al. 2002). Furthermore, sponges feed exclusively from the water column, but the nutrients they expel are available to both benthic and pelagic organisms, likely resulting in a net transfer of nutrients to the benthos.

Sponges have the ability to filter large quantities of water on a nearly constant basis, up to 50,000 liters per day per liter of sponge biomass (Weisz 2006). Assuming a conservative pumping rate estimate of 5000 L L^{-1} d^{-1} (Weisz 2006) and biomass density of 2.1 L m^{-2} (Wulff 2006), a sponge population could filter a layer of water 10.5 m deep on a daily basis. Changes in nutrient and organic matter concentrations by sponges could therefore impact much of benthic boundary layer, and nutrients from metabolic waste could elevate nutrient concentrations near the benthos (Pile 1996). As discussed above, increases in nutrient concentrations can have multiple direct and indirect consequences. Therefore, the density of sponge populations could broadly impact reef ecology.

Microbial communities in sponges

Although consumption and remineralization of particulate organic matter may be an important part of the reef N budget, the presence of microbial communities in some species could cause their role to be much more complex than simple heterotrophy. In some sponges, microbial biomass can comprise up to 50% of the animal volume (Rutzler 1981). More

commonly, microbial densities are on the order of coastal sediment (Freidrich et al. 1999) and can be 2-4 orders of magnitude higher than the surrounding water column (Hentschel et al. 2003). The sponge species that host large communities of microbial organisms were dubbed "bacteriosponges" by Reiswig, or "high microbial abundance" (HMA) or, conversely, "low microbial abundance" (LMA). Hentschel (2006) defines HMA sponges as those species that host dense microbial communities that are different from water column microbiota, whereas LMA sponges contain microorganisms at lower densities with community composition similar to that found in the water column.

Microbial communities in HMA sponges can be very diverse, including cyanobacteria (Vacelet 1971, Rutzler 1985, Vicente 1990), eukaryotic algae, heterotrophic bacteria, chemosynthetic bacteria (Corredor et al. 1988, Vacelet et al. 1996, Diaz and Ward 1997, Hentschel et al. 2006), dinoflagellates (Garson et al. 1999), and archaea (Preston et al. 1996, Pape et al. 2006). Recent studies on sponge microbial community composition indicate that there may be groups of related microorganisms that are sponge-specific (Hentchel et al. 2003, Friedrich et al. 1999, and references therein). Gene sequences that were obtained from different species of sponge, collected from different oceans, cluster closely together (Hentschel et al. 2006). This supports the hypothesis that these bacterial assemblages are stable consortia that may be specialized for this niche.

The success of certain coral reef organisms can be partially explained by efficient recycling of nutrients, either in the form of intimate symbioses with autotrophs (Muscatine and D'Elia 1978, Hawkins and Klumpp 1995), or more general mutualistic relationships among autotrophs and heterotrophs (Spott 1996). The nature of the relationship between sponges and their microbial communities has long been a topic of debate (Vacelet 1969,

Vacelet and Donadey 1977, Wilkinson et al. 1984). The apparent stability of the community composition over time and space (Hentschel et al. 2006), and the apparent health of the sponges they inhabit suggest that the association is not detrimental to the sponge. Cyanobacteria are usually localized in the ectosomal (outer) layer of tissue (Simpson 1984 and references therein) in order to have access to light. Microorganisms have also been observed in specialized vacuoles or "cyanocytes" (Wilkinson 1978), and, in some cases, integrated into spongin (Rutzler 1985). These specialized niches could suggest a degree of adaptation by sponges to accommodate microbial partners. Furthermore, sponges recognize and avoid consuming members of their own microbial community (Wilkinson et al. 1984). Although there is substantial observational evidence for symbiosis, the relationship is still not completely understood at a mechanistic level. The nutritive benefits for the microbes are evident: the filtering action of the sponge concentrates organic particles and metabolic waste products that provide a source of carbon and nutrients for uptake or chemosynthesis (Corredor et al. 1988, Diaz and Ward 1997, Pile et al. 2003 Muller et al. 2004). The benefits for the sponge host are less clear. In sponges with photosynthetic communities, fixed carbon appears to be transferred to the host in the form of glycerol (Wilkinson 1979). Sponges that host cyanobacteria have higher growth rates in light compared to shade (Wilkinson and Vacelet 1979, Frost and Williamson 1980) and restructure their morphology to enhance flow when transplanted to deep sites (Maldonado and Young 1998). These studies suggest that contributions from photosynthetic microbes are important for sponge nutrition. Some sponge-hosted bacteria produce biologically active compounds that are thought to be beneficial to sponge health (Bewley et al. 1996) or to reduce predation by fish (Dunlap and Pawlik 1988). Furthermore, stable isotopic C and N ratios from sponge skeletal material

suggests that there is a transfer of both C and N from the microbial community to the sponge host (Weisz 2006). It is unproven whether this transfer occurs by consumption of microbial cells or by transfer of dissolved compounds, but Wilkinson (1978) found that lysis of resident microbial cells was rare. Therefore, the associations between HMA sponges and their microbial communities appear to be stable and mutually beneficial.

Biogeochemical processes hosted by sponges

As discussed above, the rapid pumping rates of sponges indicate that large volumes of seawater pass through the dense microbial communities in sponge tissue on a nearly constant basis. This concentration of fresh POM and microbial life into an organic matrix of sponge tissue presents an exciting potential for rapid rates of biogeochemical processes and perhaps novel associations between microbial types. Sponges have been shown to host many microbially-mediated processes, including photosynthesis (Wilkinson 1983), and methane oxidation (Vacelet et al. 1996). Furthermore, some sponges have been shown to have anoxic zones in their interior tissues (Hoffman et al. 2005). In *Geodia baretti*, these zones facilitate sulfate reduction, and the oxic/anoxic gradient in sponge tissue appears to support sulfide oxidation (Hoffman et al. 2005). The concentration of oxygen in sponge interior tissues can therefore be an important control on the relative importance of aerobic and anaerobic processes hosted in the sponge (Muller et al. 2004). As oxygen concentration is likely influenced by the amount of irrigation in the sponge tissue and the pumping rate, sponge behavior may have a significant impact on the rates of biogeochemical processes performed by the microbial community. HMA sponges have been shown to have lower pumping rates than LMA sponges (Weisz 2006). Further, Reiswig (1971) observed episodic reductions in

flow that could not be explained by disturbance or by cyclic rhythms. Therefore, sponge pumping rates and sponge behavior could be an important determinant of their role in biogeochemical nutrient cycling.

Several of the processes hosted by sponges involve transformations of nitrogen (N). As they constitute a large pool of organic N, processes that control fluxes of N from sponges to the rest of the ecosystem could be substantial. Nitrogen fixation was first reported by Wilkinson and Fay (1979), though later efforts by Wilkinson to quantify this process produced variable results (1999). Nitrate release (attributed to oxidation of waste ammonia by the microbial community) was first reported by Corredor et al. (1988), and later by Diaz and Ward (1997). The body of literature on the microbial ecology and the biochemistry of sponge-hosted microorganisms continues to grow; however the rates and overall ecological significance of the chemical transformations they perform is still poorly understood.

The chapters that follow employ a combination of direct and indirect evidence in an effort to characterize the role of sponges in remineralization of organic matter, nitrification, assimilation of inorganic nitrogen, and nitrogen fixation. The direct evidence includes measurements of DIN fluxes, and manipulative experiments using isotopic tracers and chemical inhibitors, whereas the indirect evidence includes natural abundance C and N isotopic composition and pigment concentrations in sponge tissue. The potential for other processes, such as denitrification, anammox, and N_2O production is also discussed. I will begin by using a stable C and N isotope survey of sediment, suspended particulate organic matter, seagrass, and sponges as the basis for a discussion of C and N sources across environmental gradients near Key Largo, Florida. Sponge isotopic composition will further be used in combination with microalgal pigment concentrations to contrast LMA and HMA

species and generate hypotheses about microbially-mediated processes occurring in HMA sponges. The subsequent chapters will then focus on directly testing the hypotheses suggested by the stable isotope data.

Although sponges occur in diverse habitats, the research here focuses on sponges on coral reefs near Key Largo. Florida Keys reefs have undergone severe changes in recent decades, from disease (Porter et al. 2004) to bleaching events (Szmant and Gassman 1990), to more gradual changes in water quality and sedimentation (Dustan 1977, Porter et al. 1999). These changes devastated coral populations (Porter and Meier 1992) such that sponges are the dominant benthic macrofauna in some regions (Diaz and Rutzler 2001 and references therein). Therefore, it is especially important to understand the potential biogeochemical processes and potential changes in water quality associated with sponges and their microbial communities. Furthermore, if sponges are to dominate future reefs, and if they are the "organic bodies supplying the place of the reef-building polypifers" foretold by Darwin, then they are worthy, in their own right, of the utmost scientific scrutiny.

CHAPTER 2

Carbon and nitrogen sources indicated by isotopic composition of particulate organic matter and LMA sponges

Note: A portion of the sponge isotope data in this chapter and the next represent the joint efforts of our research team (Martens, Weisz, and Lindquist and Southwell). By mutual agreement, a subset of the dataset appears in this dissertation, in the dissertation of Weisz and in Martens et al., in prep. However, the data analysis presented here was done independently and the interpretations therefore do not necessarily reflect those of the other members of the research team.

Stable isotopes of C and N have been used extensively in marine environments as a tool to infer trophic relationships (Fry et al. 1984, Wada 1991, Marguillier et al. 1997, Sammarco et al. 1999), biogeochemical processes (Yamamuro et al. 1995), and nutrient sources (France et al. 1998, Heikoop et al. 2000). In coastal systems, C and N isotopic compositions of organic matter can help resolve inputs of terrestrial and marine-derived organic matter. N isotopic composition has been extensively used to indicate sources of N, especially those with distinctive isotopic composition, such as sewage inputs (Rogers 2003) and N_2 fixation (Montoya et al. 2002). In the Florida Keys, there are many potential sources of organic matter and nutrients due to the heterogeneity of the environment. Furthermore, rapid increases in population and land development in the Upper Keys have caused concern

about anthropogenic eutrophication from septic tanks and sewage injection wells (Lapointe and Clark 1992).

Analysis of the $\delta^{13}C$ and $\delta^{15}N$ values of organic matter has the potential to elucidate some of the sources of nutrients and organic matter in the Florida Keys. However, stable isotopic compositions of sedimentary organic matter may be altered by diagenetic processes and may not reflect that of fresh organic matter. Analysis of suspended particulate organic matter (SPOM) is more likely to reflect that of fresh organic matter sources, however repeated intensive sampling is required because the isotopic composition of SPOM can fluctuate over space and time (Evans et al. 2006). Because of their near-constant filtration of the water column, sponges could be a valuable time-integrated indicator of fresh organic matter sources (Ward-Paige et al. 2005). For this reason, a survey of stable isotopic compositions of sponges, sediment, and suspended particulate organic matter (SPOM) from various habitats around Key Largo was conducted. These habitats included patch reefs surrounded by seagrass beds, the outer fringing reef, and one shipwreck site, plus several sites in Florida Bay that were located near seagrass beds or mangroves (Figure 2.1).

In this chapter, I will discuss 1) the sources of C and N indicated by stable isotopic compositions of particulate organic matter from Flordia Bay and sites near the outer reef tract near Key Largo, and 2) sources of C and N indicated by the stable isotopic composition of LMA sponges. In this dissertation, I will refer to sites between the Florida Keys and the mainland as “bayside”, and to sites seaward of the Keys as “oceanside.” Sponge stable isotopic composition could be affected by the internal microbial communities they host. Because LMA sponges have low densities of microorganisms of similar community composition as the water column, their stable isotopic composition is unlikely to be

significantly altered by microbial activity. Therefore analysis of sponge stable isotopic composition in this chapter is limited to LMA sponges, as they should most faithfully record organic matter sources. Chapter 3 discusses HMA sponges and the potential transformations of C and N by their internal microbial communities.

Methods

Sites

The sites in this survey cover a wide range of habitats, from those near the Florida mainland, to sites 12 km offshore (Figure 2.1), and with depths that range from 1 m to 35 m. The bayside sites include some that are surrounded by nearly continuous mangrove islands (e.g., Grouper Creek, Little Buttonwood Sound, Jewfish Creek), and those that are more open to the rest of the bay, located near broad seagrass beds (e.g. Cowpen Cut, Cotton Key, Nest Key, Tern Key). The oceanside sites include patch reefs in Hawk Channel that are surrounded by seagrass beds (Tavernier Rocks, Three Sisters, Triangles), sites on the outer reef tract (Conch, Pickles), and one shipwreck (Duane).

Collection and Storage

Sponge tissue samples, seagrass, and sediment samples were collected by SCUBA divers in 2000-2003. Small pieces of sponge (about 3 cm^3) were excised with a razor blade. The top 1 cm of sediment was collected using 50 mL centrifuge tubes or modified 60 mL disposable syringes. The samples were kept submerged in seawater at *in situ* temperatures until brought back to the lab and frozen at -20^{o}C. Seagrass blades were rinsed with fresh water, but epiphytes were left in place. Seawater for SPOM samples was obtained by

pumping water (using a plastic manual bilge pump) from approximately 2 m above the sea floor into acid-washed (10% HCl) and DI-water rinsed polypropylene containers. The samples were first passed through a 39 μm mesh nitex sieve with to remove large particles, then filtered on Whatman GF/F glass fiber filters (nominal pore size 0.7μm) using vacuum filtration. The filters were frozen (-20^{o}C) and the volume of filtrate recorded.

Preparation and analysis

Sponge tissue, sediment, seagrass, and filters were dried in a lyophilizer. Sediment was then acidified using 10% HCl, centrifuged, and rinsed with deionized water. This liquid acidification technique was necessary due to the high carbonate content of the sediment (Hedges and Stern 1984). Sponge tissue, seagrass and acidified sediment samples were then homogenized with a ceramic mortar and pestle and weighed into tared silver boats. Homogenized sponge samples, seagrass, and filters were vapor-acidified using concentrated HCl overnight and dried at 80^{o}C for 1 hour. Filters were then wrapped in tin sheets and the sponge and sediment sample boats were closed and crimped. Samples were analyzed for C and N isotopic composition on a Carlo-Erba Elemental Analyzer coupled with a Finnigan Matt 252 Mass Spectrometer. Accuracy of isotopic analyses was determined by comparison with an acetanilide reference material with known isotopic composition. Standard deviation of isotope measurements was equal to or better than 0.2‰ for δ^{13}C and 0.3‰ for δ^{15}N.

Statistical treatment

The δ^{13}C and δ^{15}N values were grouped by site and species (for sponges) or type (for particulate organic matter). For SPOM, there was insufficient replication (due to sample loss

during analysis) for comparison of individual sites. Therefore, sites were grouped using cluster analysis of $\delta^{13}C$ and $\delta^{15}N$, performed in the statistical software package Jmp, version 2.0. For sponges, the 3 species with the widest geographical distribution were used for analysis of spatial trends. Sponges were absent at many of the bayside sites, and at bayside sites where there were sponges present, the species were different from oceanside sites. Therefore, only sponges from oceanside sites were used in spatial analysis (the complete dataset of sediment, SPOM, and seagrass appears in Appendix A).

Means for each species (or organic matter type) were analyzed using a generalized linear model in Jmp 2.0, and the factors in the regression included location, depth, and species. Spatial trends were not analyzed in bayside sediment because of the complex geography. Significance levels for regression slopes and comparison of means were determined at $p < 0.05$. For site location, both the distance from shore and the distance to the 20m isobath were considered, and the factor with the best statistical fit was used for the purpose of graphical presentation of the data. The 20 m isobath was chosen because it is the approximate boundary between outer, fringing reefs and Hawk Channel, where isolated patch reefs are located. Comparison of means was performed using a 2-tailed t-test with unequal variances. Site means for SPOM, sediment, and sponges were plotted on a GIS map using ArcGIS. Seagrass coverage data in the ArcGIS plots were provided by Jim Fourqurean.

Results

Particulate organic matter

SPOM

SPOM samples from Three Sisters were obtained directly after a storm during which sediments were resuspended, and were therefore excluded from comparisons with other sites. SPOM samples (not including Three Sisters) ranged from -23.0 to -17.5‰ for $\delta^{13}C$ values and 1.54 to 5.08‰ for $\delta^{15}N$ values (Figures 2.2 and 2.3, data not shown for C:N). Cluster analysis grouped oceanside sites and bayside sites together, with the exception of Jewfish Creek and Grouper Creek, which grouped more closely with oceanside sites. Due to their unique geographical locations, Jewfish and Grouper Creek were therefore considered as a third group (hereafter called Creek sites). Mean SPOM isotopic compositions for oceanside, bayside, and Creek sites appear in Table 2.1.

Seagrass

Seagrass (primarily *Thalassia testudinum*) had a mean C/N of 25.5 $\pm$ 3.3, a mean $\delta^{13}C$ value of -7.9 $\pm$ 1.1‰, and a mean $\delta^{15}N$ value of 2.1 $\pm$ 0.8 ‰ (Table 2.1).

Oceanside sediment

For oceanside sediment, , $\delta^{13}C$ values ranged from -13.2 to -17.0‰ (Figure 2.4), $\delta^{15}N$ values ranged from 1.0 to 2.6‰(Figure 2.5), and C/N ranged from 8.2 to 9.9 (Figure 2.6). For all 3 variables ($\delta^{13}C$, $\delta^{15}N$ and C/N), the regression variable with the highest adjusted R^2 value and lowest p value was water column depth, therefore sediment data were plotted with respect to depth. All three variables had significant trends with respect to depth, with C/N

decreasing (Figure 2.6), $\delta^{13}C$ decreasing (Figure 2.7), and $\delta^{15}N$ increasing (Figure 2.8) towards deeper sites offshore. Complete regression statistics appear in Appendix B.

Bayside sediment

Bayside sediment isotopic compositions showed a broader range than oceanside sediments, from -12.8 to -17.9‰ for $\delta^{13}C$ values and 2.5 to 6.1‰ for $\delta^{15}N$ values (Figures 2.4 and 2.5). C/N ratios ranged from 8.8 to 12.9. Mean C:N ratios and $\delta^{15}N$ values were generally higher than oceanside sediments (Figure 2.5 and Appendix B).

LMA sponges

Carbon isotopic composition

C isotopic composition of LMA sponges ranged from -19.4 to -13.2‰ (Figure 2.9). For the 3 LMA species with the largest distribution, (*A. compressa, C. vaginalis*, and *N. erecta*), the $\delta^{13}C$ of sponge tissue decreased with depth and distance to shore; however these variables had lower statistical significance than distance to the 20 m isobath (Figure 2.9, regression statistics in Appendix B). At the two sites nearest to shore, the $\delta^{13}C$ of LMA sponges and sediment were very similar: sponges from Triangles and Tavernier Rocks were not statistically different from the sediment. At sites farther from shore, the $\delta^{13}C$ values of LMA sponges and sediment diverged as depth and distance increased, with LMA sponges becoming more depleted in ^{13}C compared to the sediment organic carbon (Figure 2.10).

Nitrogen isotopic composition

The $\delta^{15}N$ values of LMA sponges range from 3.0 to 5.5 ‰ (Figure 2.11), and, unlike $\delta^{13}C$, the values do not echo the trends in sediment, rather they exhibit the opposite trend (Figure 2.12). As a group, the $\delta^{15}N$ values of LMA sponges showed significant spatial trends, with the strongest regression factor being distance to shore. However, when the species are analyzed individually, *A. compressa* was the only species in which the relationship was significant. *A. compressa* consistently has the highest $\delta^{15}N$ of the 3 species, and *C. vaginalis* is consistently the lowest. The differences between the species are greatest at the nearshore sites, and the values converge at the deep sites (Figure 2.13).

Discussion

Suspended particulate organic matter

Sites that were in close proximity tended to have similar $\delta^{13}C$ and $\delta^{15}N$ (Figures 2.2 and 2.3). Oceanside sites had $\delta^{13}C$ values typical of marine phytoplankton (Fogel and Cifuentes 1993), whereas bayside sites had the higher $\delta^{13}C$, perhaps due to inputs of C from the abundant seagrass beds. Because particles larger than 39 μm were excluded from the sample, seagrass-derived C was most likely in the form of very small detritus or heterotrophic bacteria. Most of Florida Bay is very shallow, and so the relative importance of benthic organic matter sources may be greater than in deeper sites. SPOM $\delta^{13}C$ of Creek Sites was significantly lower than Bayside Sites, possibly due to the influence of the dense mangrove islands that surround these sites (Figure 2.1). Mangrove organic matter was not

analyzed in this study, however mangrove leaves are typically around -27‰ (Rodelli et al. 2004), and could thus contribute to the lower $\delta^{13}C$ values of SPOM.

Sediments

Oceanside sites

Sedimentary organic matter at oceanside sites exhibit spatial trends in C/N ratios and in C and N isotopic composition that appear to be related to local sources of organic matter (Figures 2.6-2.8). The higher C/N ratios near shore could be interpreted as inputs of terrestrial material, however this is not consistent with the high $\delta^{13}C$ values of the sediment organic matter. The more likely source is seagrass or other benthic macroalgae. Seagrass in Hawk Channel, dominated by *Thalassia testudinum*, was found to be enriched in ^{13}C (-7.9 ‰ $\pm$ 1.1) and had a high C/N (25.5 $\pm$ 3.3), in agreement with previous work (Fourqurean et al. 2005, Behringer and Butler 2006). Also, $\delta^{13}C$ of sediment appears to correlate, at least qualitatively, with seagrass density (Figure 2.4).

The relative importance of seagrass and benthic macroalgae as sources of organic matter could largely be controlled by the depth, as shortening the water column decreases the volume of overlying seawater in which planktonic productivity may take place. Depth also affects light exposure to the benthos, and seagrasses have a higher minimum light requirement compared to phytoplankton (Duarte 1991). Light availability decreases exponentially with depth according to the equation: $\frac{I_x}{I_o} = e^{-cx}$, where I_o = incident light, I_x = light at depth x, c = the light attenuation coefficient, and x is depth. Light attenuation coefficients in this region are typically between 0.1 and 0.2 m^{-1} (Boyer and Briceno 2005).

Using the more conservative 0.1 m^{-1}, these values translate to 74% of incident light at 3 m, versus 37% of incident light at 10 m depth. Therefore, even relatively small changes in depth can significantly affect the relative light availability at these shallow sites, and by extension the potential benthic productivity. The light attenuation coefficient will depend on the amount of light-absorbing substances in the water column, and for seagrass, light availability can also depend on epiphyte coverage (Drake et al. 2003).

The -13 to -15‰ $\delta^{13}C$ of sediment organic matter in this region was interpreted by Behringer and Butler (2006) as evidence that macroalgae such as *Laurencia sp.* dominate the input of organic C to the sediments because *Laurencia* was found to have a $\delta^{13}C$ value most similar to that of the sediments. However, this isotopic composition could also be achieved by equal inputs of SPOM (~ -21‰) and seagrass (-8 to -9‰, Fourqurean et al. 2006, and this study). Given the extensive seagrass meadows that surround these patch reefs, and the approximately 4‰ difference in $\delta^{13}C$ values of LMA sponges at sites with and without seagrass beds (Figure 4), the latter model seems more likely. Furthermore, the high rates of leaf production (3.3 g (dry wt) m^{-2} d^{-1}) and turnover (2.7% d^{-1}), support the contribution of significant amounts of seagrass-derived organic C to the system (Martinez-Daransas et al. 2005). In the interest of conciseness, I will therefore focus on seagrass in the following discussion, although it should be acknowledged that other forms of benthic algae may also contribute some organic carbon to the system.

Thalassia testudinum analyzed from these sites had an average C/N ratio of 25, consistent with a larger *T. testudinum* survey (Fourqurean et al. 2005); in contrast, the C/N of plankton is generally about 7 (Redfield, 1958). Therefore, plankton should be a better source of organic N than seagrass: if benthic primary producers and phytoplankton are contributing

equal amounts of organic C, then plankton are contributing about 80% of the N. In such a case, the $\delta^{15}N$ of the sediment might then be expected to reflect mostly that of the planktonic input. However, the comparatively greater reactivity of algal cells compared to seagrass (Pease 2000, Hee et al. 2001) may preclude their preservation in sediments. Also, the C/N ratios in the sediments of this survey (9.06 ± 0.45) were lower than those expected from an area where macrophytes are important sources of refractory organic matter. This may be due to the high rates of N_2 fixation associated with sediments in seagrass beds (Welsh 2000, and references therein) and with seagrass epiphytes (Iizumi and Yamamuro 2000). The low $\delta^{15}N$ values of sediment (Figure 2.8) at shallow sites support this hypothesis. Sedimentary N, therefore, appears to be dominated by a combination of refractory organic matter and newly fixed N and probably does not reflect the N isotopic composition of fresh labile organic matter such as planktonic cells.

Bayside sites

It is more difficult to reconcile sources of organic matter in bayside sites because of the heterogeneity of this area. The sediment from Florida Bay appears to reflect mixtures of organic matter from seagrass, mangroves, and phytoplankton sources, and possibly alteration by biogeochemical processes. Sites near the mainland and Creek Sites exhibit lower d13C values compared to sites in the middle of Florida Bay. Although the lower values are not in the range associated with terrestrial input (the lowest $\delta^{13}C$ value is -17.5‰ at Alligator Bay), the rest of the sites in Florida Bay are considerably more enriched in ^{13}C (up to -13.1‰ at Swash Key). The comparitively low $\delta^{13}C$ values of sites near the mainland and Creek Sites suggest that these sediments receive significant OC inputs from a ^{13}C depleted source. The

most likely source of this ^{13}C depleted carbon is terrestrial plants, (e.g., mangroves and other trees) owing to the proximity to land (Figure 2.4). Sites that are remote from large land masses have $\delta^{13}C$ values similar to shallow offshore sites, possibly also due to seagrass inputs. Bayside sediment $\delta^{15}N$ values are all higher than oceanside sites, particularly in the northern section (Figure 2.5). This pattern suggests input from ^{15}N-enriched terrestrial sources at those sites, which are nearer to the mainland. If the higher sedimentary $\delta^{15}N$ values do reflect input of terrestrial organic matter, then intermediate values in the southern bayside sites (Cotton Key and Cowpen Cut) suggest that there is less terrigenous input there, or that this input becomes diluted by other sources. Indeed, there is likely to be more advective exchange with oceanside waters at southern bayside sites because of their proximity to the straights near Tavernier Key (Figure 2.1). Without greater sampling resolution, it is not possible to delineate specific sources of organic matter to this region of Florida Bay. However, it is still important to characterize the isotopic composition of organic matter from Florida Bay because of its potential to be transported to coral reefs offshore. The isotopic data suggest that organic matter from Florida Bay is enriched in ^{15}N and ^{13}C relative to oceanside sites, and that input of this organic matter from the bay to sediments and sponges is reflected in their higher $\delta^{13}C$ and $\delta^{15}N$ values.

$\delta^{13}C$ of LMA sponges

Because sponges feed on SPOM, it might be expected that they exhibit isotopic compositions similar to that source. However, LMA sponges in this survey were more similar to sediments, and showed spatial trends in $\delta^{13}C$ values (Figure 2.10) that were similar to those in the sediment. The dissimilarity between LMA sponges and SPOM could be due

to the limited amount of SPOM data available, as the isotopic composition of this pool of organic matter is expected to vary seasonally. The similarity between sponges and sediment indicates that the hypothesized seagrass C source contributes to sponge organic C as well as sediment. The $\delta^{13}C$ values of sponges and sediments are most similar at shallow near-shore sites, suggesting significant benthic-pelagic coupling there (Figure 2.10). At deeper off-shore sites, the $\delta^{13}C$ values of the LMA sponges become more depleted in ^{13}C relative to the sediment organic matter, possibly reflecting a proportionally greater input of planktonic carbon. The precise mechanism of sponge-sediment coupling is unknown. The shallow depth of Hawk Channel (<6m) and the smaller grain size of the sediment compared to deeper sites could facilitate sediment resuspension in this environment, potentially including organic detritus and heterotrophic bacteria. However, the frequency of such resuspension events is unknown, and sponges have been observed to slow or cease pumping during physical disturbances such as high turbulence or high turbidity (Reiswig 1974). Thus, it is difficult to predict the potential for sponges to consume detrital C in this way.

Sponges could also acquire seagrass-derived C via the DOM exuded by this organism (Ziegler and Benner 1999a). Although LMA sponge cells probably cannot assimilate DOM directly, efficient consumption by heterotrophic water column bacteria (Ziegler and Benner 1999a) converts seagrass-derived C into particulate form that could be available to sponges. In this model, sponges consume heterotrophic bacterioplankton whose main C source is seagrass DOM. Therefore, I will still refer to this source as "seagrass-derived C" even though the mechanism of delivery is via the heterotrohic microplankton. The absence of seagrass beds at deep sites would therefore remove not only ^{13}C-enriched source material, but

also the mechanism for benthic-pelagic coupling. Thus, even if seagrass detritus is transported to sites remote from the living beds, it may not be available to sponges.

$\delta^{15}N$ of LMA sponges

Although $\delta^{13}C$ values of LMA sponges echoes the spatial trend in the sediments, $\delta^{15}N$ values do not (Figures 2.11, 2.12). Nearshore LMA sponges are enriched in ^{15}N relative to offshore, although at the species level this trend is only significant for *A. compressa* (Figure 2.13). The three LMA species shown in Figure 13 all have different $\delta^{15}N$ values, and these differences between species could be due in part to differences in species-specific food habits. *C. vaginalis* preferentially retains *Synechococcus* and *Prochlorococcus* type bacteria (Pile 1997). *Synechococcus* has been shown to fix N_2 (Mitsui et al. 1986); therefore, if this bacterium comprises a greater proportion of *C. vaginalis* diet than other sponges, this preference could explain the lower $\delta^{15}N$ values of *C. vaginalis* relative to the other two LMA species. Although there is no information on the feeding preferences of the other two species, most sponges are generalist filter feeders.

As the sediments probably do not reflect fresh, labile sources of N, the decoupling of sediment and sponge $\delta^{15}N$ values is not surprising. Trophic enrichment of N by sponges could explain the ~ 3‰ difference in the sediment and sponge values, but it cannot explain the opposing trends. As discussed above, the -13 to -15‰ $\delta^{13}C$ values of sponges and the decrease in $\delta^{13}C$ with depth suggest that seagrass and/or other benthic macrophytes are an important source of C, however, it is uncertain whether seagrass could be an important source of organic N because of its high C/N.

As discussed above, the high $\delta^{13}C$ of LMA sponges suggests consumption of seagrass-derived C, and that this consumption could occur via heterotrophic water column bacteria that feed on seagrass DOM. However, these bacterioplankton may also assimilate dissolved nutrients from the water column, thus achieving a C isotopic composition similar to seagrass and an N isotopic composition similar to phytoplankton. Given the high and variable C/N ratio of seagrass DOM (14-81 Ziegler and Benner 1999b), it is likely that this DIN uptake occurs to some extent. Therefore, the C and N sources for bacterioplankton (and thus LMA sponges) may be decoupled: although the C content of heterotrophic water column bacteria may be largely seagrass-derived, the N content is likely derived from a combination of seagrass DOM and dissolved inorganic nitrogen.

Both heterotrophic and autotrophic plankton may partially reflect sources of inorganic N in the water column. Thus, sponges filtering these organisms should reflect these sources as well, and so the higher $\delta^{15}N$ of LMA sponges near shore could be interpreted as ^{15}N enrichment in inorganic N sources near land. Although some studies have suggested that elevated sponge $\delta^{15}N$ values indicate land-derived pollution (Ward-Paige et al. 2006), elevated $\delta^{15}N$ values do not automatically prove anthropogenic N loading. While "heavy" N can be derived from terrestrial sources (e.g. sewage, runoff; Rogers 2003), it may also be derived from recycled N in Florida Bay's water column and sediments. Sediment and SPOM data show that particulate organic matter (both sedimentary and suspended) from Florida Bay are enriched in ^{15}N compared to oceanside samples, and that this could be due to a combination of source material from land, but also perhaps from biogeochemical processing of N in the sediments and water column. Advective exchange between the bay and the ocean can occur via the straights near Tavernier Key, and to the Southwest (Figure 2.1). Sponges

from Tavernier Rocks, which is the reef closest to shore and closest to large straights connecting the Atlantic Ocean and Florida Bay, have the highest $\delta^{15}N$ values. The differences in $\delta^{15}N$ values here are small, and not as high as values typically associated with anthropogenic N loading, however, the pattern of elevated $\delta^{15}N$ at Tavernier Rocks is echoed in each of the 3 LMA species. Therefore, the elevated $\delta^{15}N$ of sponges at nearshore sites could reasonably be explained by transport of ^{15}N-enriched N from Florida Bay through the straights near Tavernier Key.

Although it is tempting to conclude that land-derived N is the cause of the spatial trend in LMA $\delta^{15}N$ values, there is not yet enough evidence to do so given the complexity of this environment. The role of the seagrass beds and the associated microbial communities cannot be overstated. Fourqurean et al. (2005) showed that seagrass nutrient ratios and isotopic composition are dynamic, both seasonally and spatially. Therefore, the interactions between seagrass, water column bacteria and phytoplankton, along with the potential for nutrient limitation during peak productivity, limit the ability to use $\delta^{15}N$ alone to conclusively evaluate sources of N in this environment. Nevertheless, it is interesting that the spatial trend in $\delta^{15}N$ of LMA sponges is not reproduced in the surface sediments, SPOM, or seagrass data. Because they incorporate fresh organic material into more refractory compounds such as spongin, LMA sponges may provide a unique isotopic record of highly reactive organic matter such as algal and bacterial cells.

Conclusions

Although C sources (e.g. macroalgae) could be significant, the spatial trends in $\delta^{13}C$ values of sediment and LMA sponges appear to be related to the presence or absence of seagrass beds. Significant contribution of seagrass-derived organic carbon is suggested by the high $\delta^{13}C$ values of both bayside and shallow oceanside sediments, in agreement with previous work on seagrass influence on sedimentary OC $\delta^{13}C$ values (Papdimitriou et al. 2005). A simple source mixing model with SPOM and seagrass as potential sources suggests that, in Hawk Channel, approximately half of sedimentary organic C and sponge biomass C is derived from seagrass and half from SPOM. Elevated $\delta^{15}N$ values in bayside sediments are likely caused by input from terrestrial sources. However, because the $\delta^{15}N$ values of organic matter in these sediments are likely to be strongly influenced by processes such as N_2 fixation, and remineralization, they are not reliable indicators of N isotopic composition of allochthonous organic matter sources.

In contrast to sediments, LMA sponges incorporate fresh organic matter sources and generally reflect their dietary organic matter source, though there are slight differences between species. *A. compressa* exhibits the strongest spatial trend in $\delta^{15}N$ values among LMA sponges, and it has a broad distribution. It could therefore potentially serve as a bio-indicator species for changes in organic matter sources. This sponge exhibits increasing $\delta^{15}N$ values with decreasing distance to shore, and the highest values of this sponge (and the other 2 LMA species) occur near areas of exchange with Florida Bay. While this spatial trend is consistent with transport of ^{15}N-enriched N from Florida Bay towards the reef tract, it is not possible to determine the ultimate source of that N (e.g. runoff, sewage seepage) without

additional data. For example, higher resolution sampling could help elucidate spatial patterns within the Bay. Nevertheless, this study has demonstrated that LMA sponges, unlike sediments, record the carbon and nitrogen isotopic composition of fresh, labile organic matter. Because sediments are not useful indicators of allochthonous N input, sponges may be especially useful for investigating changes in N sources over time and space.

Tables

			$\delta^{15}N$, ‰		$\delta^{13}C$, ‰	
Sample Type	Location	Sites	Mean	SD	Mean	SD
Sediment	Offshore	3	2.2	0.5	-16.2	0.9
Sediment	Hawk Channel	3	1.2	0.2	-13.9	0.2
Sediment	Bayside	8	4.5	1.0	-15.0	1.9
SPOM	Ocean	3	2.2	0.3	-21.3	0.6
SPOM	Bayside	5	4.0	1.2	-18.7	0.9
SPOM	Creek	2	2.0	0.6	-23.0	0.1
Seagrass	Hawk Channel	2	2.1	0.8	-7.9	1.1
LMA sponges	offshore	4	4.1	0.2	-17.7	1.0
LMA sponges	Hawk Channel	3	4.5	0.2	-14.4	0.6

Table 2.1. Summary of particulate organic matter C and N isotopic compositions from Florida Bay, Hawk Channel, and the offshore reef tract. SD = standard deviation.

Figures

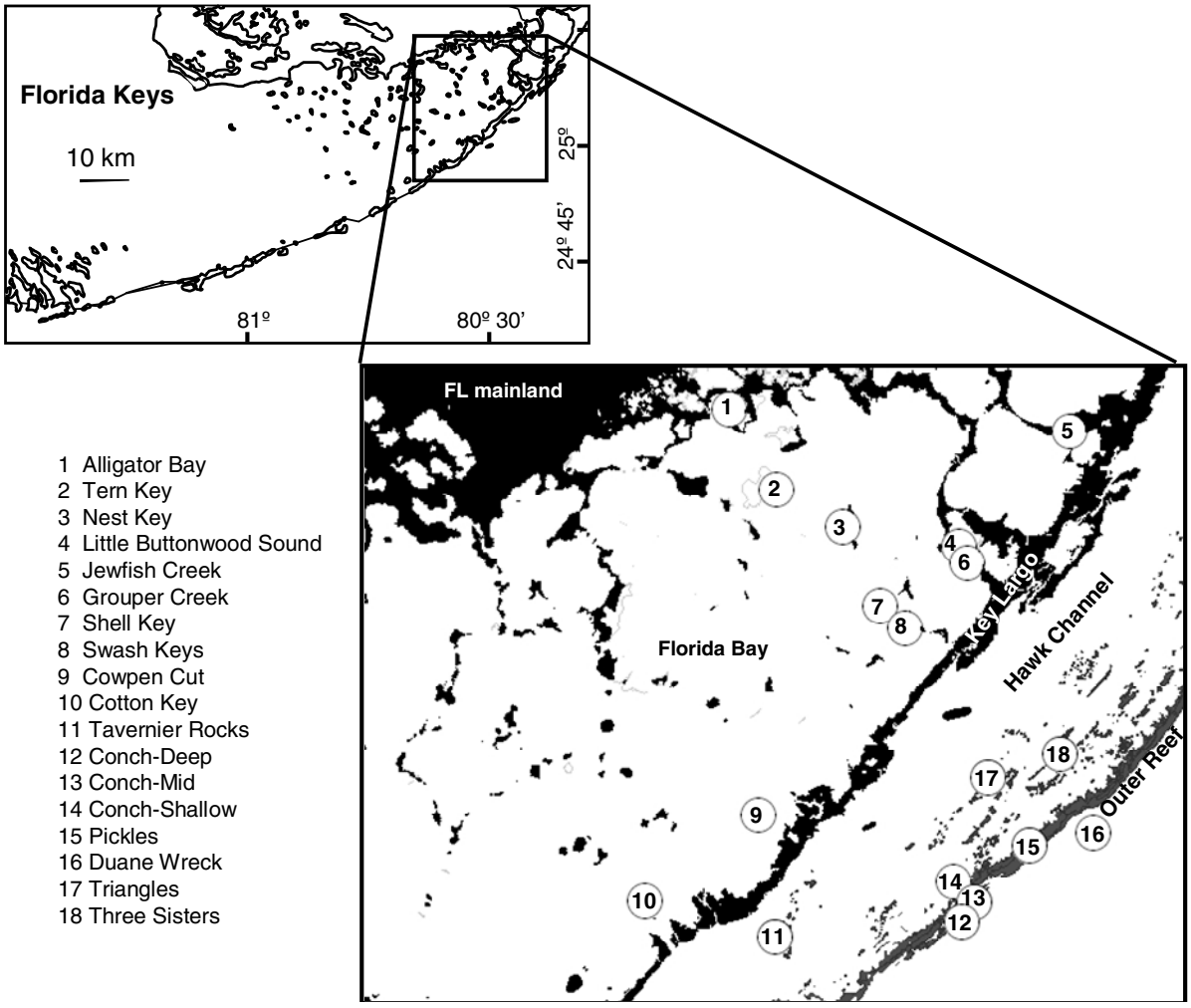

Figure 2.1. Map of sites.

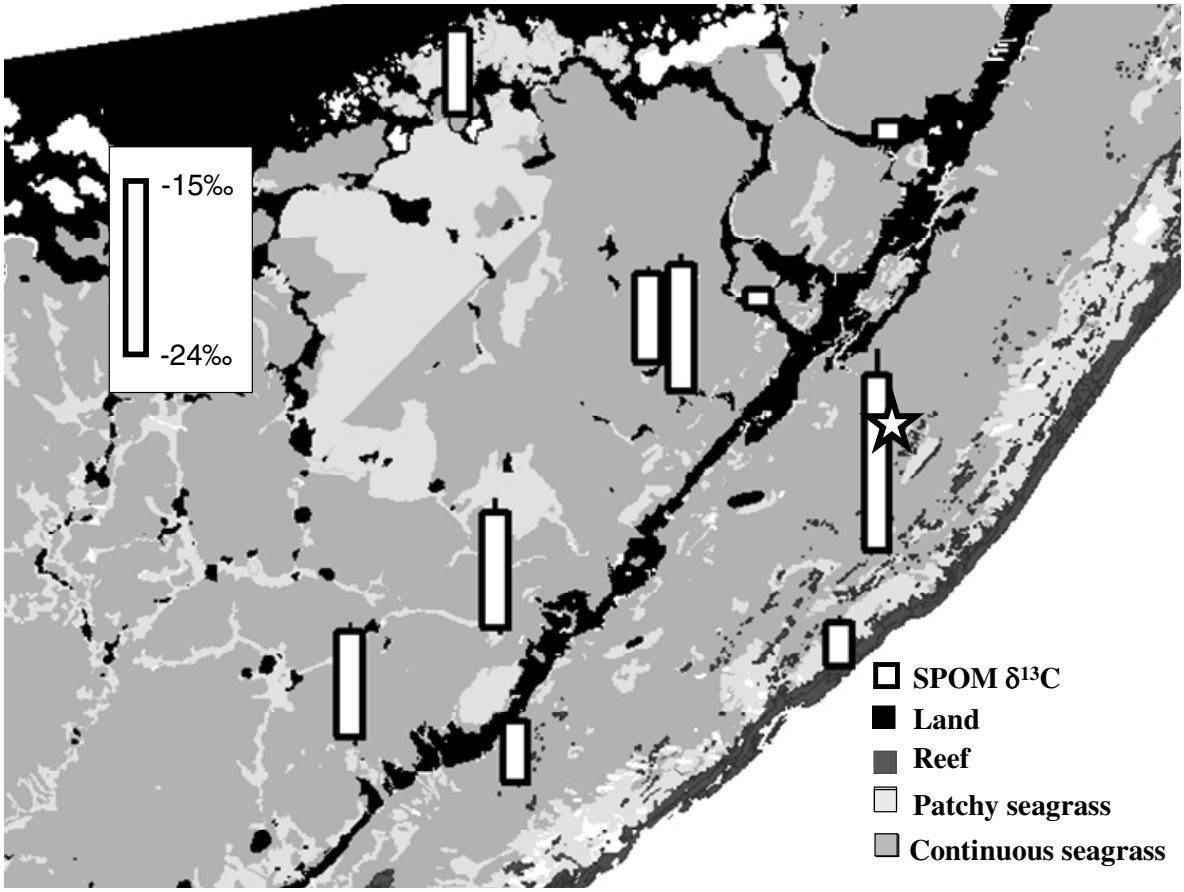

Figure 2.2. SPOM $\delta^{13}C$. Star denotes Three Sisters data point, which was collected after a high wind event. Error bars are standard error. Seagrass coverage data in this plot and those that follow were provided by Jim Fourqurean.

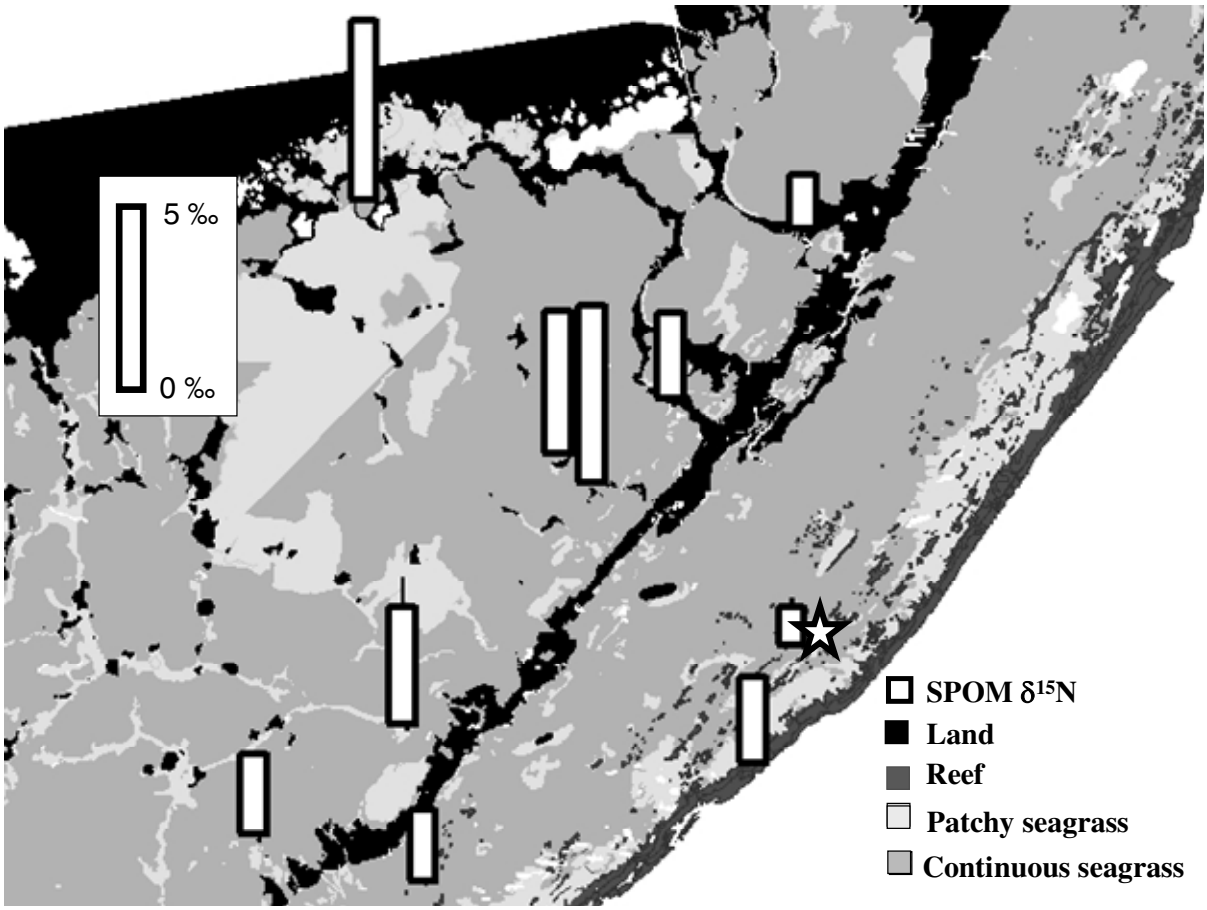

Figure 2.3. SPOM $\delta^{15}N$. Star denotes Three Sisters data point, which was collected after a high wind event. Error bars are standard error.

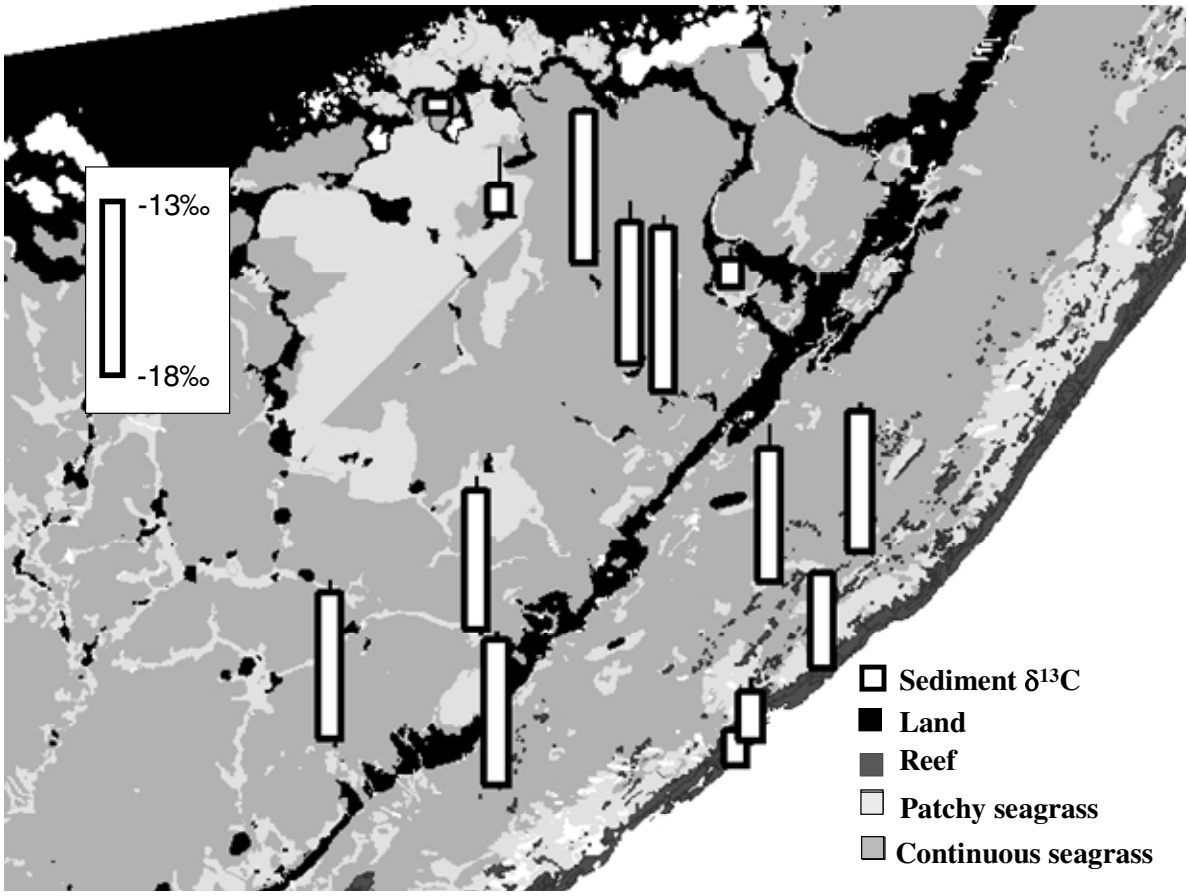

Figure 2.4. $\delta^{13}C$ values of sedimentary organic matter. Error bars are standard error.

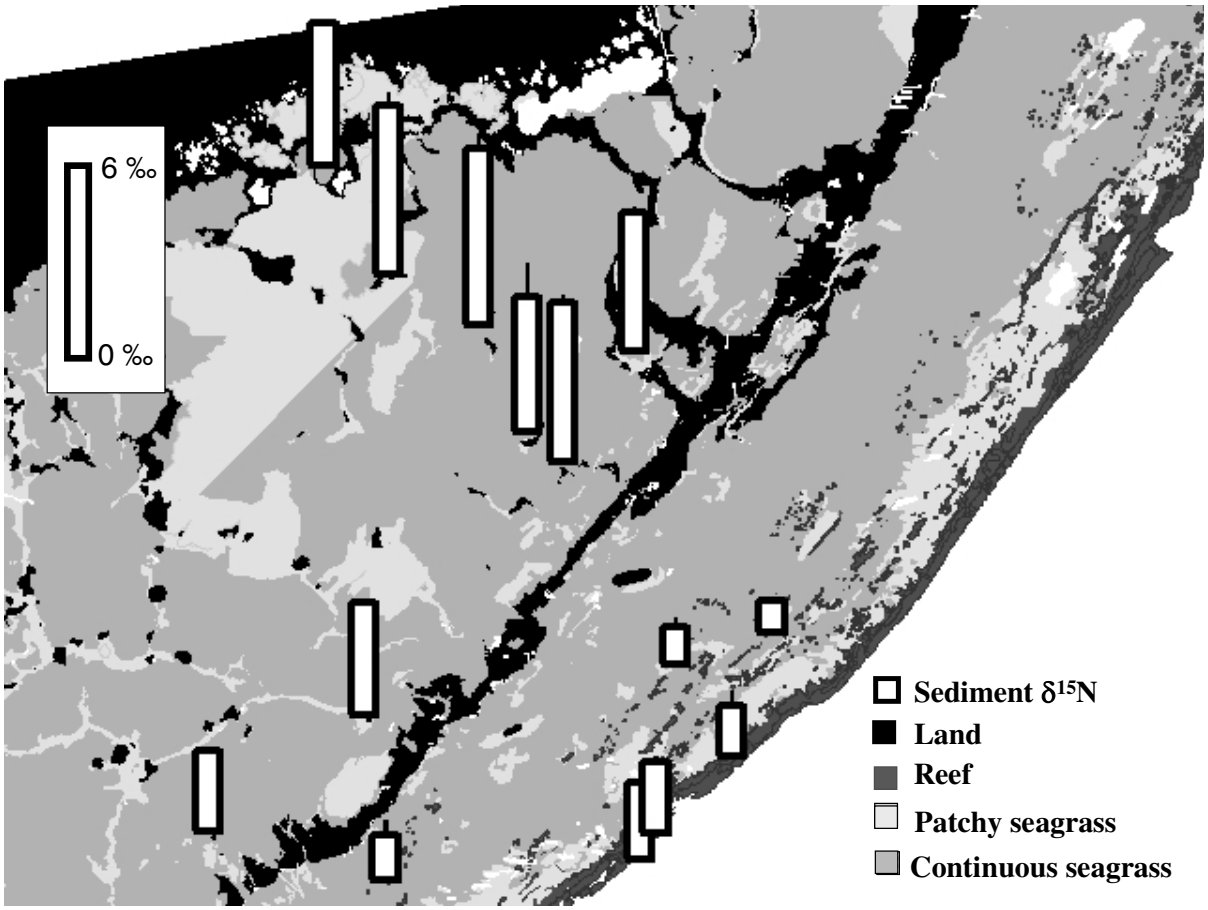

Figure 2.5. $\delta^{15}N$ values of sedimentary organic matter. Error bars are standard error.

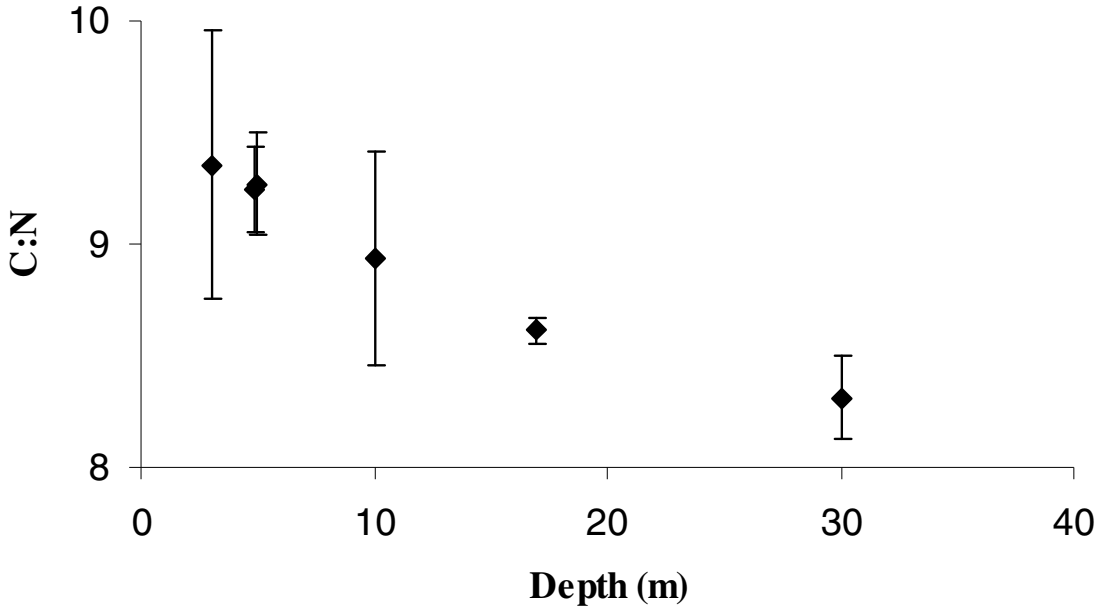

Figure 2.6. C:N ratios in oceanside sedimentary organic matter. Error bars are standard deviation.

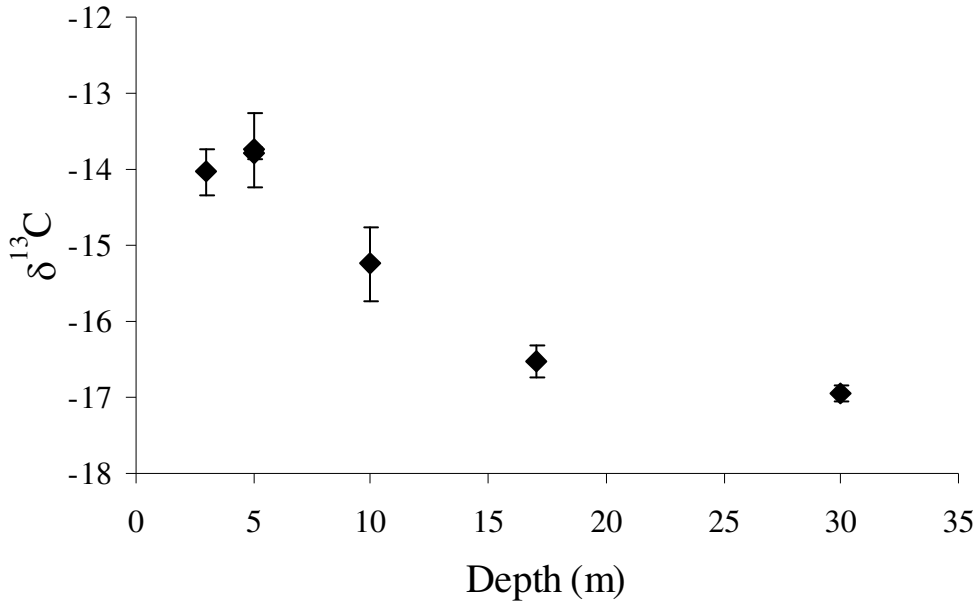

Figure 2.7. Sedimentary organic carbon $\delta^{13}C$ values in oceanside sediment versus depth. Error bars are standard deviation.

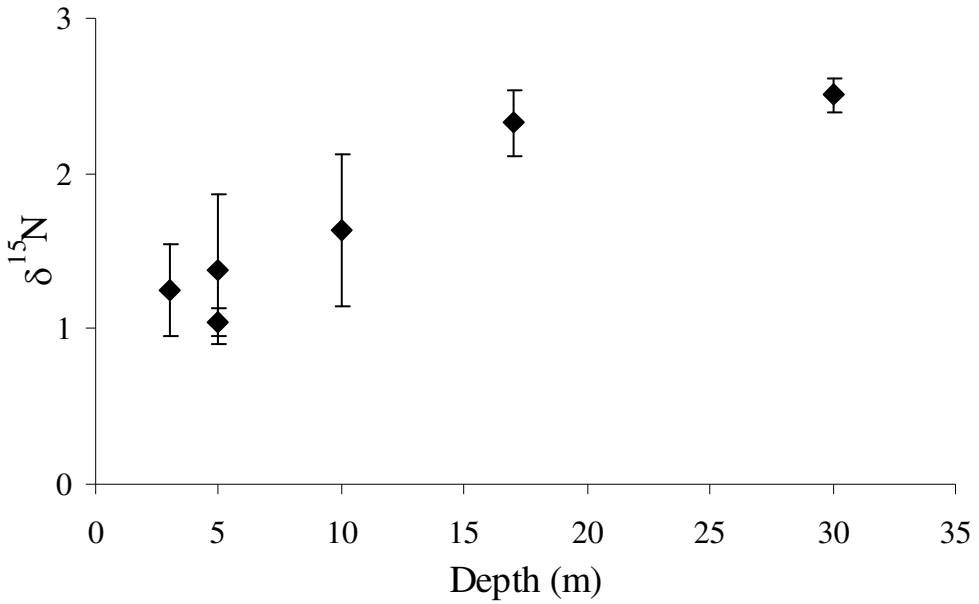

Figure 2.8. $\delta^{15}N$ values of sedimentary organic matter in oceanside sediment. Error bars are standard deviation.

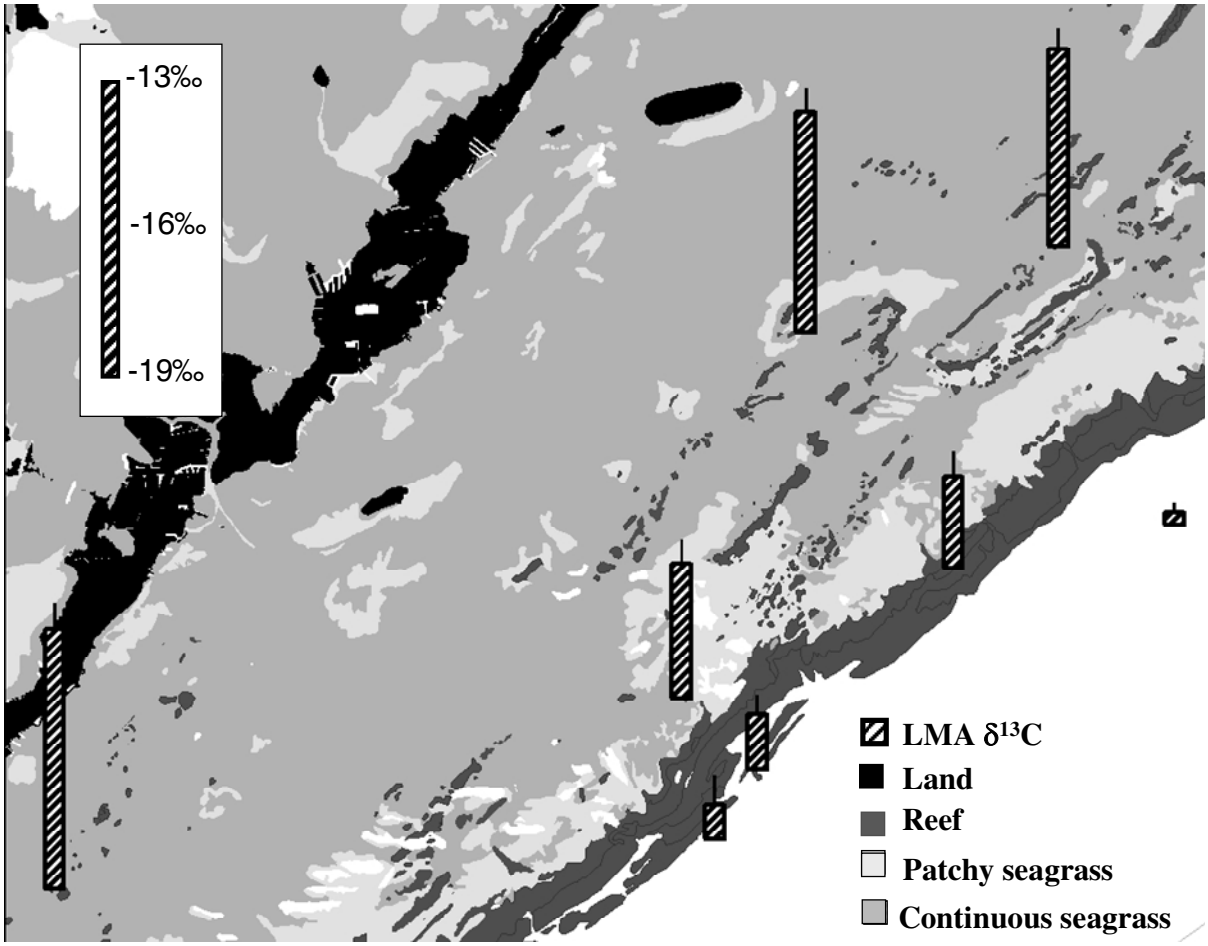

Figure 2.9. LMA sponge $\delta^{13}C$ values. Error bars are standard error.

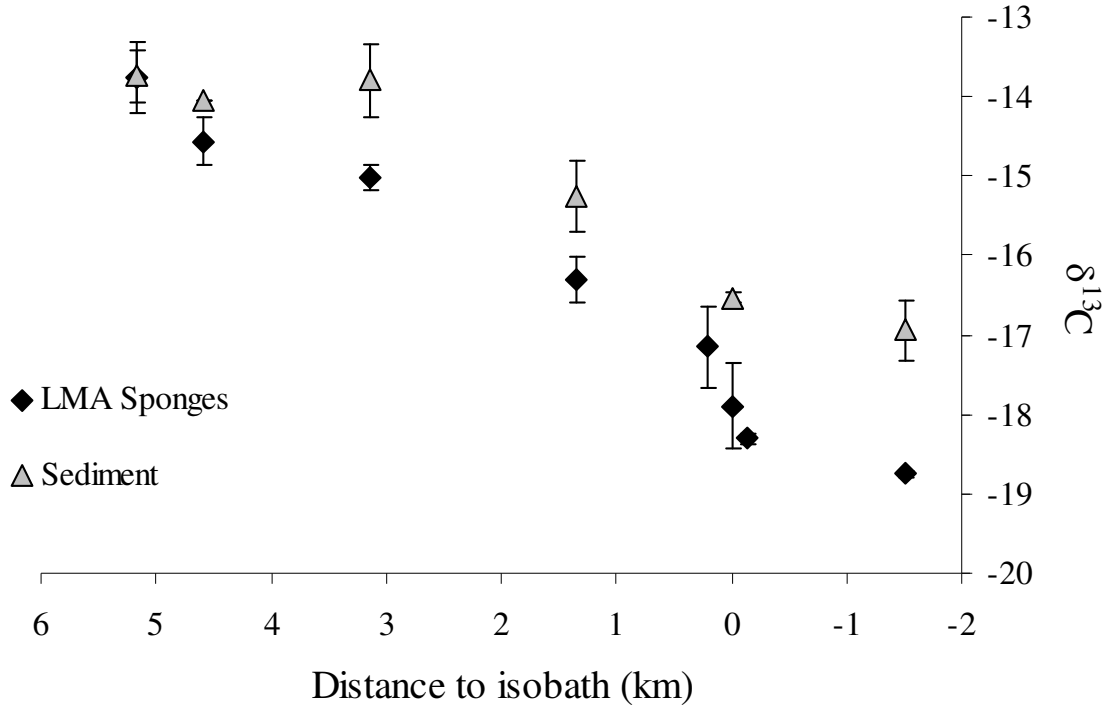

Figure 2.10. Sediment and LMA sponge $\delta^{13}C$ values. Error bars are standard deviation.

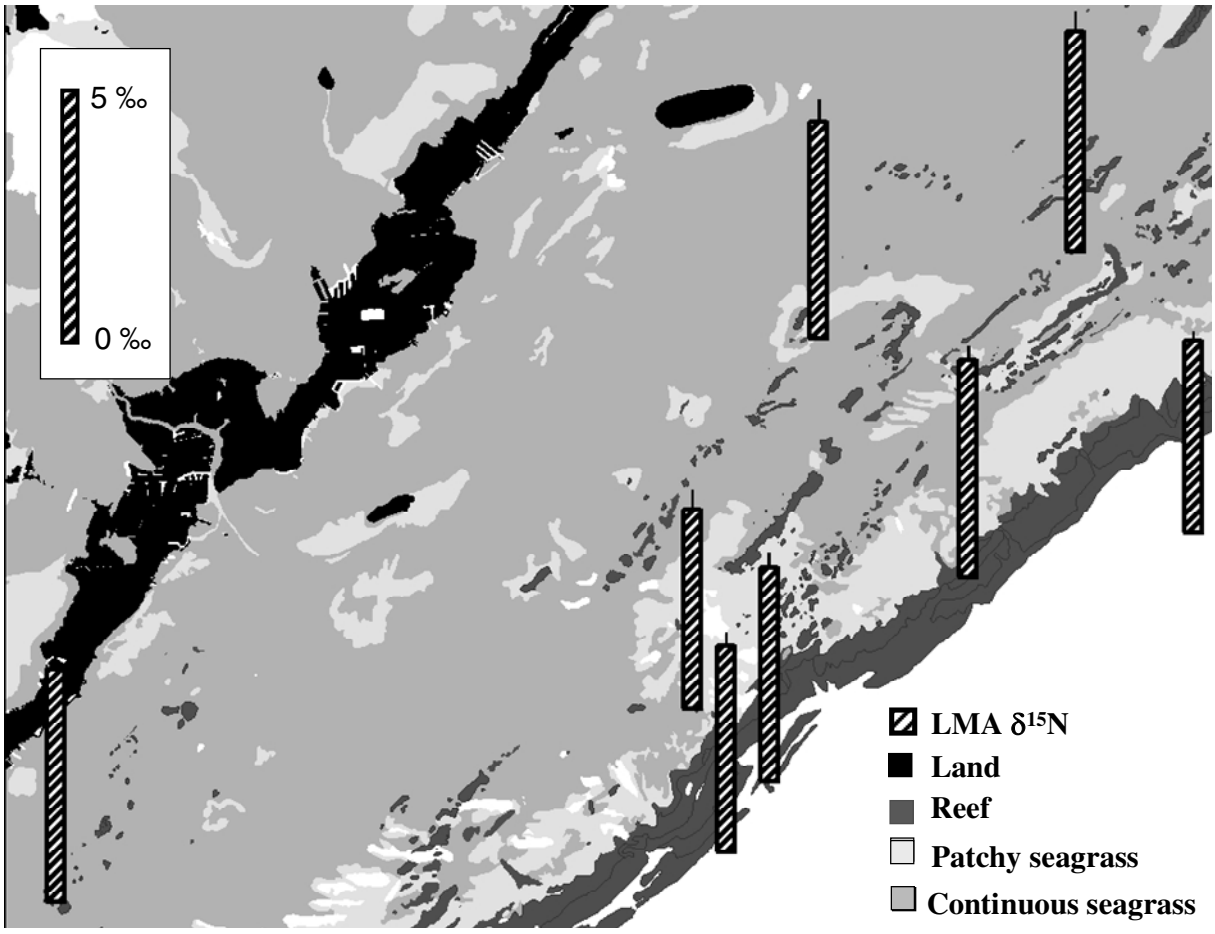

Figure 2.11. LMA sponge $\delta^{15}N$ values. Error bars are standard error.

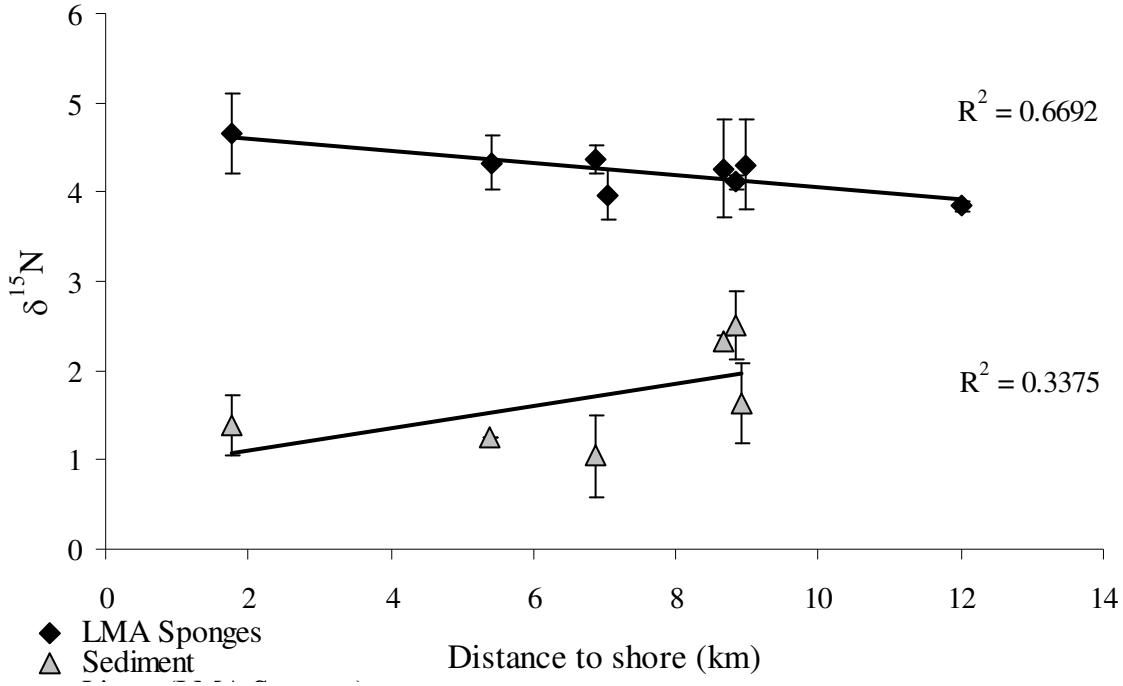

Figure 2.121. $\delta^{15}N$ values of LMA sponges and sediment. Error bars are standard deviation.

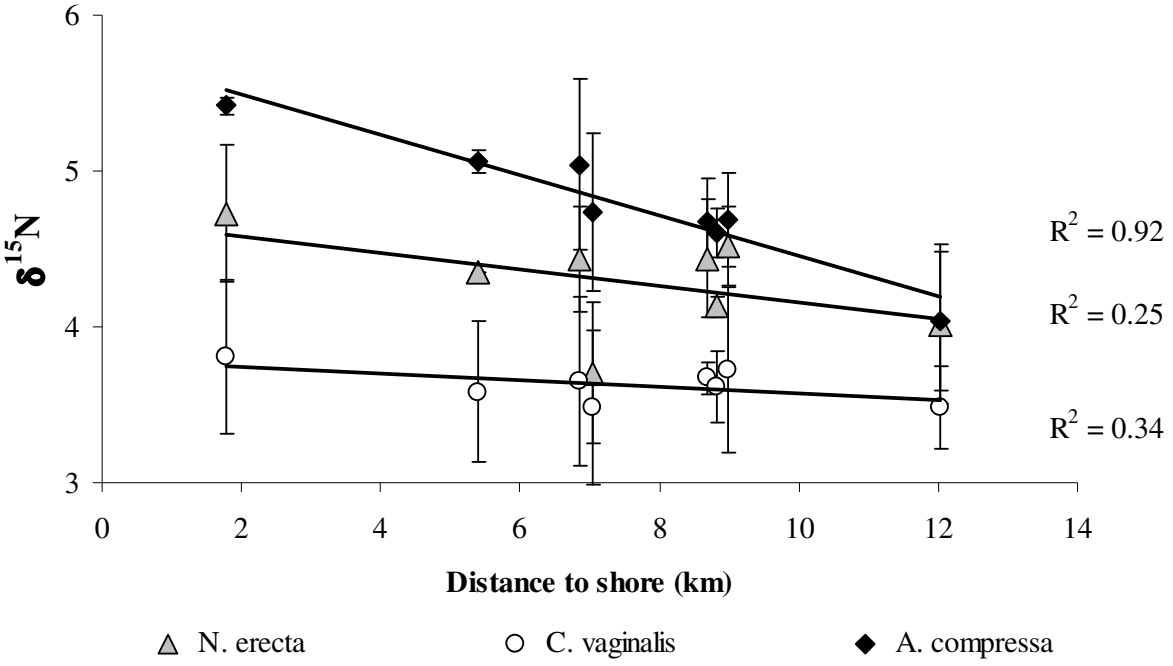

Figure 2.13. Species-specific mean $\delta^{15}N$ values of 3 LMA sponge species. Error bars are standard deviation.

CHAPTER 3

Influence of internal microbial communities on sponge C and N isotopic composition

The previous chapter demonstrated that the stable isotopic composition of LMA sponges can reflect local organic matter sources, potentially including information about labile organic matter sources not recorded in sediments. Other studies have used sponge nitrogen isotopic composition to infer nitrogen source inputs (Ward-Paige et al. 2005, Behringer and Butler 2006), but without determining the potential effects of internal microbially-mediated processes on sponge isotopic compositions. Sponge-hosted microbial communities have been shown to perform a variety of biogeochemical processes involving transformations of C and N (Wilkinson and Fay 1979, Wilkinson 1983, Corredor et al. 1988, Diaz and Ward 1997) and these processes could affect sponge $\delta^{13}C$ and $\delta^{15}N$ values. Cyanobacteria and other photosynthetic microorganisms are common among HMA sponges (Chesire and Wilkinson 1991), and may perform not only photosynthesis (Wilkinson 1983), but also uptake of DIN (Davy et al. 2002, Pile et al. 2003), and possibly N_2 fixation (Wilkinson and Fay 1979, Wilkinson 1999). Heterotrophic microorganisms, which are also present in sponges (Hentschel 2003, Richelle-Maurer et al. 2003), may be capable of DOM or DIN uptake (Reiswig 1981, Davy et al. 2002, Pile et al. 2003, Yahel et al. 2003) as well as N_2 fixation under appropriate environmental conditions (hypoxia and anoxia). HMA sponges

are thus much more complex than LMA sponges in terms of potential sources of C and N. Therefore, interpretations of organic matter sources based on stable isotope compositions of HMA sponges are complicated at best, and studies that completely ignore potential effects of the resident microbial community on sponge $\delta^{13}C$ and $\delta^{15}N$ values may lead to incomplete conclusions about organic matter sources.

Although the potential processes involving cycling of C and N by HMA sponges are complex compared to their simpler LMA cousins (Figure 3.1), this characteristic also makes HMA sponges interesting subjects for scientific research. Furthermore, untangling the isotopic influence of organic matter sources versus internal processing is possible if the source effects are well known. Here, this requirement can be fulfilled by LMA sponges: because microbial processing in LMA sponges should be negligible, their isotopic composition should reflect only net source effects. This chapter therefore uses differences in $\delta^{13}C$ and $\delta^{15}N$ values of HMA and LMA bulk tissue to generate hypotheses about internal microbially-mediated processes in HMA sponges. These hypotheses are then supported with analysis of photosynthetic pigments in sponge tissue, comparisons of sponges from different habitats and light exposure, and comparisons of different sponge tissue types. Using this approach, it may be possible to isolate the effects of various C and N cycling processes and determine their relative importance.

Methods

Sample collection

Bulk sponge tissue

Collections for bulk sponge tissue were made simultaneously with those described in the previous chapter (see Chapter 2 for site descriptions and methods).

Light/dark comparison

Sponge samples were collected from Cowpen Cut, Grouper Creek, and the Aquarius underwater laboratory (at Conch Reef, map appears in Chapter 2). These sites were chosen because they have individuals of the same species inhabiting both sunlit and cryptic habitats in close proximity. At Cowpen Cut and Grouper Creek, sponges were collected from the tops and shaded undersides of mangrove prop roots. At Aquarius, sponges were collected from the top and underside of the artificial structure.

Endosome/ectosome comparison

Sections of sponge tissue were excised at Three Sisters Reef, Pickles Reef, and Conch Reef, (locations described in Chapter 2), and were kept in ambient seawater until returned to the lab. The outer 2 mm of tissue was then removed with razor blades and both portions were stored separately. All samples were obtained, processed and frozen (-20^{o}C) the same day.

Pigment samples

Sponge tissue (visually free of epiphytes) was collected for pigment analysis from Conch Reef in January 2006. Samples were kept on dry ice until brought back to the lab, and then frozen at -80°C.

Sample preparation and analysis

Stable isotopes

Sponge tissue was lyophilized and ground with a mortar and pestle until homogenous. Subsamples were weighed (~3 mg) into silver cups, then vapor-acidified overnight to remove calcium carbonate (Hedges and Stern 1984). Samples were then dried for 1-2 hours at 80 °C and the cups were folded closed and compressed. Samples were analyzed on a Carlo-Erba CHN analyzer coupled to a Finnigan MAT Con-Flo Isotope Ratio Mass Spectrometer.

Chlorophyll-a

Chlorophyll-a analysis was done according to EPA method 445.0 revision 1.2. All pigment extraction was conducted in a darkened lab. The outer 2 mm of sponge tissue was thawed, drained of excess water on a paper towel, and extracted with 4 mL of 100% acetone in a 15 mL centrifuge tube. This was modified from 90% acetone in the EPA method to account for the water content of the sponge (which was approximately 12%, Appendix C). The sponge was macerated in the acetone with a glass stirring rod and sonicated (15 pulses) while submerged in an ice bath. The centrifuge tubes were then covered with parafilm and allowed to incubate in the dark at -20°C overnight. The sponge-acetone slurry was then re-suspended by gentle stirring, and the extract was filtered through a 0.45 μm nylon syringe filter into a borosilicate test tube. The fluorescence of the extract was measured on a Turner

Designs model TD-700 with a chlorophyll-a optical kit. The raw fluorescence was then compared to known standards (obtained from Turner Designs) and the extracted sponge tissue was then dried and weighed.

Accessory pigments

The ectosome (outer 2 mm) of the sponge was thawed and drained of excess water on a paper towel. Sponge tissue was then extracted and analyzed following the method of Leavitt and Hodgson (2001). Briefly, the tissue was placed in 2.5 mL centrifuge tubes with 1 mL of extraction solvent (acetone:methanol:water, 80:15:5). The samples were sonicated (15 pulses) with a probe sonicator while submerged in an ice bath, then stored at -20^{o}C overnight. The extract was centrifuged and the supernatant was filtered with a 0.22 μm syringe filter. The pigment extract was analyzed by Matt Waters on a Shimadzu HPLC system with a photodiode array detector. Pigments were separated on a single reverse phase column following the mobile phase and time sequence of Leavitt and Hodgson (2001). Pigments were identified using retention times of known standards and pigment specific spectra recorded by the detector. Pigment concentrations are expressed as μg pigment/g sponge tissue (dry weight) and calculated by comparing peak areas against standards of known concentration. Following the extraction, the sponge tissue was then dried and weighed.

Pycoerythrin

Phycoerythrin was extracted following the method of Viskari and Colyer 2003. Briefly, sponge samples were extracted in 4 mL of asolectin (0.3% w/v) and Chaps (3% w/v)

in deionized water (chemicals were obtained from Sigma-Aldrich). Due to its limited solubility in aqueous solutions, the asolectin was first dissolved in diethyl ether and then dried under a nitrogen flow until nearly dry. Samples were submerged in ice water, sonicated with a probe sonicator in the presence of combusted sand, and then refrigerated overnight at 4^{o}C. The supernatant was then filtered (0.45 μm nylon syringe filter) and analyzed by florescence using a Turner Designs TD-700 fluorometer fitted with a phycoerythrin optical kit. Fluoresence was compared to a known standard of phycoerythrin obtained from Turner Designs. All reagents, samples, and standards were used within 24 hours of preparation.

Statistical analysis

Analysis of light/dark samples was done by comparison of means, with a 2-tailed t test with unequal variance, and significance was determined at $p < 0.05$. Endome/ectosome samples were first grouped by species, and then a paired t-test was performed ($p < 0.05$).

Spatial analysis

As with LMA sponges (Chapter 2), the three HMA species with the broadest site distribution were chosen for spatial analysis. The $\delta^{13}C$ and $\delta^{15}N$ of these sponges were analyzed with a generalized linear model in Jmp version 2.0.

Results

Pigment concentrations

Chlorophyll-a in the outer 2 mm of sponge tissue ranged from 0-600 μg g^{-1} (dry weight) of sponge, with 8 species containing significant amounts of chlorophyll-a (Figure 3.2). Results from HPLC and phycoerythrin analyses show that algal pigment composition is generally consistent among the species tested, and that phycoerythrin, zeaxanthin, chlorophyll-a, and β-carotene are the most abundant pigments (Figure 3.3). For the three species with elevated pigment concentrations, the ratio of zeaxanthin to chlorophyll-a was not significantly different, but the ratio of β-carotene to chlorophyll-a and phycoerythrin to chlorophyll-a was significantly different for all 3 species (Figure 3.4). Chlorophyll-b was present in small quantities in *A. compressa*, *I. strobilina*, and *N. erecta*, whereas it was absent in *X. muta*, *I. felix*, and *A. cauliformis* (data not shown). Peridinin was not detected in any species (data not shown).

Isotopic composition of HMA sponges

The $\delta^{13}C$ values of HMA sponges ranged from -19.2 to -13.3‰, similar to the overall range for LMA species (Figure 3.5). Some HMA species also had $\delta^{15}N$ values similar to the LMA group, however a subset of HMA species had significantly lower $\delta^{15}N$ values (Figure 3.5). In general, HMA species means showed a greater inter- and intra-species variance in $\delta^{15}N$ values and less variance in $\delta^{13}C$ values relative LMA species. Because of the large inter-species variability, I avoided analyzing HMA sponges as a group, and instead analyzed each species separately.

Spatial Analysis

For the 3 HMA species (*I. felix, A. cauliformis*, and *I. strobilina*), the most significant factors for both $\delta^{15}N$ and $\delta^{13}C$ values was distance to the 20 m isobath (see Appendix B for statistical results of regression analysis). Like sediments and LMA sponges (Chapter 2), the $\delta^{13}C$ values were positively correlated with distance (landward) from the isobath (Figure 3.6). *A. cauliformis* $\delta^{13}C$ values were lower than the LMA sponges at shallow sites, and the two means converged at deeper sites. For *I. felix,* $\delta^{13}C$ values were lower than LMA sponges at shallow sites, but higher at deep sites. *I. strobilina* $\delta^{13}C$ values were higher than LMA sponges at all sites.

All 3 HMA species in the spatial analysis were among the group with significantly lower $\delta^{15}N$ values compared to the LMA group. *A. cauliformis* and *I. felix* had significant spatial trends in $\delta^{15}N$ values, for which distance from the isobath was the strongest regression factor (Figure 3.7). In contrast to the LMA sponges, *A. cauliformis* and *I. felix* $\delta^{15}N$ values increased in the offshore direction. *I. strobilina* had no significant spatial trends in $\delta^{15}N$ values. Unlike LMA sponges, no HMA species had a significant trend with respect to $\delta^{15}N$ values and distance from shore.

Light/Dark comparisons

I. variabilis and *C. nucula* collected from the top of mangrove roots (well-lit) had significantly lower $\delta^{13}C$ values compared to those collected from the cryptic locations (Figure 3.8). *I. variabilis* and *C. nucula* from the tops of mangroves at Cowpen had higher

$\delta^{15}N$ values than shaded individuals, but there was no significant difference for the *C. nucula* collected at Grouper Creek. Although pigment concentrations were not measured in these samples, the shaded individuals were observed to be much paler than individuals collected from sunlit locations. Neither $\delta^{13}C$ nor $\delta^{15}N$ values of *I. birotulata* was significantly different for light-exposed versus shaded individuals.

Ectosome/endosome comparisons

All species except for *I. strobilina* had significant differences between ectosome and endosome $\delta^{13}C$ values ($\Delta\delta^{13}C_{ecto\text{-}endo}$), and the magnitude of the differences ranged from -0.4 to 1.6‰ (Figure 3.9). In *X. muta* and *A. cauliformis*, this difference appears to decrease with depth. The differences in $\delta^{15}N$ values of sponge ectosome versus endosome ($\Delta\ \delta^{15}N_{ecto\text{-}endo}$) ranged from -1.2 to 1.1‰ (Figure 3.10). *A. cauliformis* was the only species to exhibit significant $\Delta\ \delta^{15}N_{ecto\text{-}endo}$ and there was no significant depth trend.

Discussion

Pigment concentrations

Although there is a wealth of genetic information on the community composition of microbial populations in sponges, there is relatively little data on abundance of specific cell types. This paucity of data is not entirely surprising, given that these measurements often rely on microscopy or fluorescence *in situ* hybridization, labor intensive efforts that may or may not provide information about cell function. In contrast, pigment analysis is a rapid way to assess the relative abundance of photosynthetic cells, and secondary pigments may provide

additional information about cell types (Jeffrey et al. 1997). Given the small amount of literature devoted to photosynthetic pigments in sponges, this work has necessarily been exploratory in nature. Because very little is known about sponge microalgal pigment content, the results presented here are used simply to identify those species harboring significant photosynthetic communities and to speculate about microalgal abundance and community composition. These data expand the number of sponge species for which chlorophyll-a data is available from 10 to 26, and presents the first (to my knowledge) information on microalgal-derived phycoerythrin and carotenoid concentrations in sponge tissue.

Chlorophyll-a survey

Half the species tested contained a significant amount of chlorophyll-a in the outer 2 mm. According to a recent biomass survey, these 8 species represent 77% of the sponge biomass on Conch Reef (Chapter 4). The presence of chlorophyll a was generally consistent within a genus, except for *Ircinia*, of which *I. campana* and *I. felix* contain chlorophyll-a whereas *I. strobilina* contains negligible amounts, similar to LMA sponges (Figure 3.2). It should be noted that even LMA sponges are also expected to contain small amounts of chlorophyll-a due to the retention of phytoplankton during filter feeding. The lack of chlorophyll a in *I. strobilina* is somewhat surprising because it was reported to harbor algal cells by Vicente (1990). However, Vicente's specimens were from Belize, and the presence of photosynthetic associates could be geographically variable (although this is not supported by Hentschel et al. 2002). Furthermore, Vicente did not report abundance of photosynthetic cells, and so the density could have been very low. The chlorophyll-a concentrations from

this study were of the same order of magnitude as those measured by Wilkinson (1983) in the Great Barrier Reef. Although Wilkinson normalized for the entire sponge mass (rather than the outer 2mm only), the comparison should not have been greatly affected because most of the species in that study were less than 5 mm thick.

Chlorophyll-a concentration has commonly been used to broadly characterize phytoplankton abundance; however there are no known estimates of cell pigment content for sponge-hosted microalgae. For phytoplankton cells, Strickland (1960) estimates 30 g C (g chl a)$^{-1}$ for nutrient-rich conditions and 60 g C (g chl a)$^{-1}$ for nutrient-poor conditions. Classifying the nutrient conditions in the sponge tissue as nutrient-rich or nutrient-poor is difficult because cells on the outermost surface of the sponge tissue may experience the nutrient-poor ambient water of the reef, whereas cells slightly deeper in the tissue may experience more nutrient-replete conditions due to heterotrophic metabolism of neighboring sponge cells. Using Strickland's two values as potential end-members and the mean C content of sponge tissue (26%, data not shown), I estimate that microalgal biomass could comprise up to 7 and 14% of organic C (for nutrient-rich and nutrient-poor conditions, respectively) in the outer 2 mm of sponge tissue. Furthermore, approximately 50% of sponge bulk organic C is spongin (Weisz, J. unpubl. data). Therefore, in terms of living cells, up to 28% of the organic C in the outer 2 mm may be from biomass of photosynthetic cells.

Accessory pigments

All sponges containing chlorophyll-a also contained significant amounts of phycoerythrin, β-carotene, and zeaxanthin (Figure 3.3). Phycoerythrin and zeaxanthin are typically cyanobacterial pigments (Jeffrey et al. 1997); thus cyanobacteria appear to be

ubiquitous across all sponges tested in this study. This is consistent with previous microscopy work showing that unicellular coccoid cyanobacteria were dominant in these three species, and in most others with photosynthetic microbes (Vicente 1990). Despite this similarity, there were significant differences in the ratios of β-carotene:chl-a and of phycoerythrin:chl-a for *X. muta, A. cauliformis*, and *I. felix* (Figure 3.4), which may suggest slightly different algal compositions in these sponges. The absence of peridinin is noteworthy because dinoflagellates (which typically contain peridinin) have been reported in some sponges (Vicente 1990).

Isotopic composition

$\delta^{13}C$ values of HMA sponges

The $\delta^{13}C$ values of all 3 HMA species decreased with increasing distance from shore (Figure 3.6), as did sediment and LMA sponge $\delta^{13}C$ values (Chapter 2). However, the slope of this spatial trend was lower in HMA species compared to the LMA spatial trend (Figure 3.6 and Appendix B). This is likely due to an additional source or sources of C that dilute the onshore-offshore gradient observed in LMA sponges and sediments. Such a source is probably available only to sponges with microbial communities, because no LMA species displayed this pattern.

Photosynthesis by the resident microbial community has been shown to be important for sponge nutrition (Frost and Williamson 1980, Cheshire and Wilkinson 1991) and could thus be a significant source of C. Bil et al. (1999) showed that sponge-hosted photosynthesis occurs via the C_3 pathway, and so photosynthate is likely to have an isotopic composition similar to tropical marine phytoplankton (-20‰ Fogel and Cifuentes 1993). However, some

primary producers fix more ^{13}C when they are limited with respect to CO_2, which could be possible for algal cells embedded in a tissue matrix, depending on the amount of irrigation by sponge pumping. However, the chlorophyll-a concentrations in the ectosome suggest that photosynthetic cells are likely outnumbered by non-photosynthetic cells. Because of this moderate density, it seems unlikely that algal cells could become CO_2 limited when surrounded by respiring heterotrophic cells.

If the $\delta^{13}C$ value of photosynthate is, in fact, similar to marine phytoplankton, this input would likely be indistinguishable from that of LMA sponges at deep sites (-18 to 19 ‰). However, the ^{13}C enriched organic matter consumed by sponges at shallow sites (Chapter 2) provides a source of organic C that is isotopically very different from the expected isotopic composition of photosynthate. Furthermore, C fixation rates are likely to be greatest where light levels are highest. Using a conservative light attenuation coefficient of 0.1 m^{-1} (Boyer and Briceno 2005), light availability decreases from 74% to 5% of incident light between 3 and 30 m, thus the potential contribution of photosynthesis should be much higher at shallow sites. Differences in C isotopic compositions between species that host photosynthesis and those that do not should therefore be greatest at shallow sites.

The spatial trends for the HMA sponges *A. cauliformis* and *I. felix* (Figure 3.6) are indeed consistent with C inputs from primary production by the photosynthetic microbial community. *I. strobilina* is an HMA sponge that does not follow this pattern, but is instead more similar to LMA sponges. However, the pigment data show that *I. strobilina* does not host significant numbers of microalgae (Figures 3.2 and 3.3), and it is therefore only reasonable that it should reflect organic C sources. *I. strobina* and *I. felix* are two HMA sponges of the same genus, and they have similar distribution and morphology; yet *I.*

strobilina lacks microalgae whereas *I. felix* does not (Figures 3.2 and 3.3). At the shallowest site where both species are found, $\delta^{13}C$ values of *I. strobilina* are ~ 1‰ higher than *I. felix*, and the values converge with depth and are not significantly different at the deepest sites (Figure 3.6). Also, the slope of the linear regression of *I. strobilina* $\delta^{13}C$ values (whether regressed with respect to depth, distance to shore, or distance to the 20m isobath) is more similar to that of LMA sponges than HMA sponges (Appendix B). The difference between the two species at the shallowest site could therefore represent the effect of photosynthate input to *I. felix*.

The hypothesized C contribution from resident photosynthesizers does not exclude the possibility of DOM as another important C source for at least some HMA sponges. Sponge microbial communities have been suggested to be capable of DOM uptake (Reiswig 1981), and this mechanism appears to provide the majority of dietary C in the HMA sponge *Theonella swinhoeii* (Yahel et al. 2003). As discussed in Chapter 2, DOM could be indirectly obtained by LMA sponges via consumption of bacterioplankton. However, the mechanism of uptake (assimilation by internally hosted microbes versus filtration of heterotrophic water column bacteria) may influence the type, amount, and/or isotopic composition of DOM that sponges are able to consume. Thus, it is possible that internal DOM uptake could have a significant (though probably limited) influence on sponge isotopic composition. Very little is known about the isotopic composition of marine DOM, and much less about DOM in coral reef waters; however, it is probably influenced by exudates from the extensive beds of seagrass in Hawk Channel (Chapter 2). This DOM has never been isotopically characterized, but it is likely to be relatively isotopically enriched, given the $\delta^{13}C$ values of the source material (~ -8 to -9‰, Chapter 2).

The $\delta^{13}C$ values of the HMA sponge *I. strobilina* are consistently higher than LMA species (Figure 3.6), and this could be related to uptake of isotopically enriched DOM; however, other HMA species exhibit much different trends relative to LMA sponges. A combination of both DOM and photosynthetic inputs may explain some of the observed differences between HMA species. For example, *A. cauliformis* hosts microalgae and has a high surface area to volume ratio, and so is likely to be strongly influenced by photosynthate input: its $\delta^{13}C$ values are consistently lower than LMA species (Figure 3.6). *I. strobilina* represents the other extreme, as it has a low surface area to volume ratio and contains no significant photosynthetic microbes, and so it would be more influenced by DOM: its $\delta^{13}C$ values are consistently higher than LMA species (Figure 6). *I. felix* represents an intermediate situation in which photosynthetic cells are present (Chapter 3), but with a low surface area to volume ratio: its $\delta^{13}C$ values are low at shallow sites (where photosynthesis is likely to be more important) and higher at deeper sites (where photosynthesis is likely to be less important) (Figure3. 6).

Without constraining the isotopic end-members in this scenario, this exercise is largely hypothetical. However, this model does provide a reasonable explanation of the data. Furthermore, the results of the stable isotope survey, the light-dark comparison and the endo-ecto comparison all present internally consistent evidence for the effect of photosynthetic microbial communities on sponge C isotopic composition. This hypothesis is also supported by the chlorophyll-a data, which demonstrate a qualitative correlation between the presence of photosynthetic microbial communities and the apparent effect of light levels on sponge $\delta^{13}C$ values. Wilkinson (1983) demonstrated a positive correlation between chlorophyll-a concentrations and photosynthesis rates in sponges, and so it could be possible to make a

rough estimate of photosynthetic rates in the outer layer of these sponges. However, attempting to extrapolate photosynthetic rates from pigment concentrations is not a useful exercise in this case because the respiration rates of these sponges are unknown. Therefore, it would not be possible to calculate the relative C contributions of heterotrophy versus autotrophy, or to estimate the net isotopic composition that would result from this balance. However, despite these limitations, the pigment data provide supporting evidence for the interpretation of sponge $\delta^{13}C$ values.

$\delta^{15}N$ in HMA sponges

Unlike the $\delta^{13}C$ data, the $\delta^{15}N$ data show the strongest trend in species, not in site location. This suggests an internal effect, perhaps related to the microbial communities hosted by the sponge. The most striking feature of this survey data is the division of HMA species into 2 groups, one with $\delta^{15}N$ values from -0.1 to 2.3‰ (hereafter called "low $\delta^{15}N$" sponges), and the other with $\delta^{15}N$ values similar to LMA sponges (3.8 to 5.8‰) (Figure 3.5). The values of the first group are lower than expected for a marine filter feeder, given the expected trophic enrichment of 3.4‰ per trophic level (Minigawa and Wada 1984). Because all of the sponges with low $\delta^{15}N$ values are HMA sponges, this phenomenon is likely related to a microbially-mediated process. Work by Weisz (2006) showed that the ^{15}N depleted N also appears in the spongin of these species, suggesting that microbial N is transferred to the sponge host. The HMA sponges with high $\delta^{15}N$ values (e.g., *X. muta, A. conifera*) may not host the process that imparts this isotopically light N, or may host an additional process or processes that obscure its evidence.

There are four possible mechanisms that could explain the ^{15}N depletion in the low δ15N sponges relative to the group of high $\delta^{15}N$ sponges: selective feeding, DOM uptake, N_2 fixation, and DIN uptake. The latter three would be performed directly by the microbial community. Selective feeding could produce ^{15}N depleted tissues if N_2 fixing plankton are preferentially retained. Wilkinson (1984) found that sponges did not retain microbes that were of the same type as their internal microbial community, so some mechanism for selective feeding by HMA sponges may exist. However, if the un-retained types of microbes are represented in the internal microbial community, then their organic matter is already well represented in the bulk sponge tissue. Furthermore, flow cytometry by Pile (1997) showed that most sponges are non-specific feeders. One of the exceptions, *C. vaginalis,* preferentially retains *Synechococcus* and *Prochlorococcus* yet it is depleted in ^{15}N by only ~ 0.5‰ compared to the overall LMA average. The N isotopic composition of *C. vaginalis* is ~ 3.5 ‰ (Chapter 2), which is approximately the value expected from an organism feeding on N_2 fixers: N_2 fixing organisms are expected to have $\delta^{15}N$ values near that of atmospheric nitrogen (0‰) and animals feed on them would thus be expected to have $\delta^{15}N$ values that are approximately 3.4‰. In contrast, some low $\delta^{15}N$ sponges have values less than 0‰. Selective feeding therefore has a limited potential for incorporating light N, and probably cannot result in $\delta^{15}N$ values <0‰.

As discussed above, DOM could be an important source of carbon for reef filter feeders, and this DOM could also provide a source of N. If seagrass-derived DON is isotopically light, it could provide a source of ^{15}N depleted N and be a mechanism for the observed low $\delta^{15}N$ values in sponge tissue. However, nothing is known about the N isotopic composition of DOM exuded by seagrass. Bulk samples of *Thalassia testudinum* collected

from Hawk's Channel in Key Largo had relatively low $\delta^{15}N$ values (1.9 $\pm$ 0.3 ‰ Chapter 2), however this is not low enough to produce the lowest sponge $\delta^{15}N$ values, given the expected 3.4‰ ^{15}N trophic enrichment. Bulk DOM from the Atlantic Ocean was found to range from 6.6 to 8.9 ‰ (Benner et al. 1997), which suggests that marine DOM is generally not a good source of isotopically light N, although seagrass DOM could be very different. Benner's (1997) study also showed that trends in $\delta^{15}N$ of DON with depth were related to biological processing of N and that high molecular weight DOM is actively utilized.

It is unclear, however, whether seagrass DOM is likely to be an important source of N because C:N ratios are generally less favorable than algal POM (C:N of seagrass-derived DOM = 14 – 81, Ziegler et al 1999b; and C:N of bulk Atlantic Ocean DOM = 15.6 – 18.1, Benner et al.1997). Remineralization of seagrass DOM by water column bacteria has been associated with a release of ammonium (Ziegler and Benner 1999b), which suggests that this organic matter could be a source of labile N. However, nitrogen from DOM would be subject to the same trophic enrichment as POM; therefore HMA sponges would need to assimilate DOM that is extremely depleted in ^{15}N in order to achieve a bulk $\delta^{15}N$ value less than 0‰.

Fixation of atmospheric N is accompanied by little fractionation (Fogel and Cifuentes 1993), thus producing an isotopic composition near zero. If this is the mechanism for low $\delta^{15}N$, then the data indicate high rates of N_2 fixation to produce bulk $\delta^{15}N$ sponge tissue that is so similar to that expected for a purely N_2-fixing organism. N_2 fixation in sponges is the focus of Chapter 6, and so will not be discussed in detail here.

Where DIN concentrations are high, uptake of ammonium and/or nitrate by bacteria selects for $^{14}NH_4^+$ and $^{14}NO_3^-$ (Fogel and Cifuentes 1993). The interior of the sponge could

be relatively high in DIN due to the remineralization of organic matter, and this nutrient-rich micro-environment could facilitate incorporation of light N. The 3 HMA species in the spatial analysis all have low $\delta^{15}N$ values, but *I. strobilina* has no significant spatial trend, whereas the other species show greater ^{15}N depletion in shallow, nearshore patch reefs (Figure 3.7). Greater light availability in shallow sites may facilitate higher rates of photosynthesis and induce more DIN uptake. Alternatively, greater availability of DIN on these patch reefs may facilitate greater partitioning of isotopes during uptake. Although the spatial trends in *A. cauliformis* and *I. felix* $\delta^{15}N$ values are consistent with a light-dependent processes, they do not indicate whether that process is N_2 fixation or DIN uptake. Also, the fact that *I. strobilina* has a low $\delta^{15}N$ values despite its lack of microalgae demonstrates that light-driven processes do not entirely control, and perhaps so not even dominate, the acquisition of isotopically light N. Heterotrophic microorganisms are also capable of assimilating DIN and fixing N_2, and could therefore be the mechanism for incorporating isotopically light N into *I. strobilina* biomass.

Nitrification is known to be hosted by some sponges (Corredor et al. 1988, Diaz and Ward 1997), and this process is discussed in detail in Chapter 5. Nitrification is not an obvious mechanism for incorporation of isotopically depleted N because it produces isotopically depleted nitrate and enriched residual ammonium (Mariotti 1981, Casciotti et al. 2003). Ammonium is more easily incorporated into biomass because nitrate must first be reduced before it is incorporated into an organic molecule; therefore, the net effect of nitrification on the N isotopic ratio of biomass is more likely to be enrichment than depletion. Ammonia oxidation and nitrification can sometimes be coupled to a gaseous loss of N, either by denitrification, anammox, production of N_2O byproduct, or nitrifier denitrification (Poth

and Focht 1985). The potential for gaseous N fluxes is discussed further in Chapters 6 and 7. However, denitrification, like nitrification, is likely to produce isotopically enriched rather than depleted biomass because this $^{14}NO_3^-$ is preferentially selected (Mariotti 1981). Nevertheless, these processes may exert significant control over isotopic fractionation of N in the sponge microbial community, and could therefore explain some of the variability in HMA $\delta^{15}N$.

Light/Dark comparisons

Comparing different sponge species can provide a great deal of information, yet there remains the possibility that inter-species differences are due to feeding preferences by certain sponges (Pile 1997). One way to overcome these potential differences, and to more directly assess the importance of light-dependent processes, is to compare individuals of the same species that are exposed to different light levels. *Ircinia variabilis* and *Chondrilla nucula* collected from mangrove roots at Cowpen Cut had significantly different $\delta^{13}C$ and $\delta^{15}N$ values depending on whether they were located on the top of the prop roots (well lit) or underneath (shaded) (Figure 3.8). *Chondrilla nucula* from Grouper Creek had significantly different $\delta^{13}C$ values, but not $\delta^{15}N$ values. *I. birotulata* does not contain microalgae, and individuals collected from the top and underside of Aquarius did not have significant difference in $\delta^{13}C$ or $\delta^{15}N$ values.

Although there are large differences in isotopic compositions at these sites (likely related to organic matter sources, see Chapter 2), they represent a "natural experiment" where the only variable that is different between the paired groups is light. Therefore, the difference between light and shaded *I. variabilis* and *C. nucula* likely reflects photosynthate

input from resident microalgae. In both species, the mean $\delta^{13}C$ values of shaded individuals are about 1‰ higher than those in the light. Both *I. variabilis* and *C. nucula* collected at Cowpen Cut had significantly different $\delta^{15}N$ values for shaded versus well-lit individuals, however those differences were of opposite sign: *I. variabilis* in the light had higher $\delta^{15}N$ values, whereas *C. nucula* in the light had lower $\delta^{15}N$ values (Figure 3.8). The species also had very different absolute $\delta^{15}N$ values, suggesting fundamental differences in N source for these two sponges.

Ectosome/endosome comparison

Analyzing sponges of the same species eliminates much potential for variability however it is still possible that individuals on the top versus the underside of mangrove roots are exposed to some different organic matter sources, (e.g. exudates from the roots themselves or from the multitude of organisms that use this cryptic habitat.) Thus, an even more specific comparison is that of the ectosome (outer 2 mm) to the endosome (interior tissue) from the same individual sponge. Because actively photosynthetic cells should be concentrated in the surface tissue of the sponge, inputs from this source should also be concentrated there.

Of the five sponges that were tested, *X. muta, I. felix,* and *A. cauliformis* contain microalgae including cyanobacteria (based on pigment evidence), *I. strobilina* is an HMA sponge but lacks microalgae, and *N. erecta* is an LMA sponge (Weisz 2006). The results show that the ectosomes of the sponges with microalgae had lower $\delta^{13}C$ values than the endosomes (Figure 3.9). The endosome and ectosome $\delta^{13}C$ values of *I. strobilina* were not statistically different, and the respective layers of *N. erecta* had a small but statistically

significant difference (Figure 3.9). The average $\Delta\delta^{13}C_{ecto\text{-}endo}$ for the three species with microalgae was $0.8 \pm 0.4‰$, which is similar to the difference between the $\delta^{13}C$ values of light and shaded *I. variabilis* and *C. nucula*, and between bulk tissues of *I. felix* and *I. strobilina* at shallow sites. The depth trend in *X. muta* and *A. cauliformis* could indicate greater abundance or activity of microalgae in shallow, well-lit sites.

A. cauliformis was the only sponge to have a significant difference between $\delta^{15}N$ values of endosome and ectosome (Figure 3.10). In this sponge, ectosome $\delta^{15}N$ values were about 0.5‰ lower, possibly due to uptake of isotopically light N in the surface tissue layer. The lack of difference between the tissue layers in the other species could be interpreted in several ways: 1) no N assimilation is occurring in the surface layer, 2) N assimilation is occurring, but assimilated N is isotopically similar to organic matter sources, 3) N assimilation is occurring, but is not limited to the surface layer, or 4) N is assimilated in the surface layer and efficiently translocated to other parts of the sponge. Although photosynthetic C fixation is probably restricted to the sponge ectosome, N assimilation can be performed by heterotrophs as well as autotrophs, and so assimilated DIN may not be concentrated at the surface. Furthermore, a comparison of spongin and cellular biomass by Weisz (2006) demonstrated that low $\delta^{15}N$ nitrogen is efficiently transferred to the sponge. Therefore, the effect of microalgal N uptake may not be detectable in the ectosome/endosome comparison because the isotopically light N is dispersed throughout the sponge.

Conclusions

Significant photosynthetic pigment was found in 8 of 16 species, representing 77 percent of biomass on Conch Reef (based on biomass data from Chapter 4). Therefore, the activity of these associated microalgal cells could influence the C and N cycling of a large portion of the sponge population. Evidence for the influence of photosynthate on sponge $\delta^{13}C$ can be seen in comparisons of 1) species with and without microalgal communities, 2) individuals of the same species under different light conditions and 3) the $\delta^{13}C$ values of the ectosome versus the endosome of individual sponges. In addition, DOM may also influence the $\delta^{13}C$ values of some sponges, however there is less evidence for this.

The morphology of the sponge may play a role in determining the importance of photosynthesis as a source of C. *A. cauliformis* has the highest surface area:volume ratio, the lowest bulk $\delta^{13}C$ values at shallow sites, and the largest difference between endosome and ectosome $\delta^{13}C$ and $\delta^{15}N$ values. In contrast, *I. felix* has low surface area:volume, intermediate bulk tissue $\delta^{13}C$ values at shallow sites, and little to no difference in $\delta^{13}C$ and $\delta^{15}N$ values of ectosome-endosome. The isotopic evidence presented here is consistent with previous work on the importance of morphology on sponge-microalgal symbiosis.

The set of processes controlling $\delta^{15}N$ values of HMA sponges appears to be quite complex. There are distinct differences in mean $\delta^{15}N$ values of bulk sponge tissue, however those differences are not consistent with any obvious characteristics (e.g., presence of microalgal community, pigment content, morphology). Spatial trends in $\delta^{15}N$ values suggest that assimilation of isotopically light N is coupled with photosynthesis in *I. felix* and *A.*

cauliformis, but not in *I. strobilina*. The mechanism for light-dependent incorporation could be N_2 fixation or DIN uptake, and none of the comparisons presented here serve to differentiate between these two processes. The two HMA species in the light/dark comparison showed opposite trends in the effect of light on $\delta^{15}N$, despite the fact that they are both HMA species and were located in close proximity. This and other results from the isotopic analysis of sponges imply that it may not be possible to generalize about N cycling by HMA sponges. Rather, the relative importance of various N sources and transformations may be specific to certain species.

HMA sponges represent a much more complicated system of biogeochemical cycling than LMA sponges. The isotopic compositions of HMA sponges in this study were affected by the same source gradients as the LMA sponges, plus there was evidence of additional internal processing of carbon and nutrients by the sponge microbial community. Although untangling the factors resulting in the bulk isotopic composition of HMA sponges may be difficult, it is potentially a more exciting, and more important task, as the chemical reactions within the sponge may be significant relative to overall reef C and N budgets.

Figures

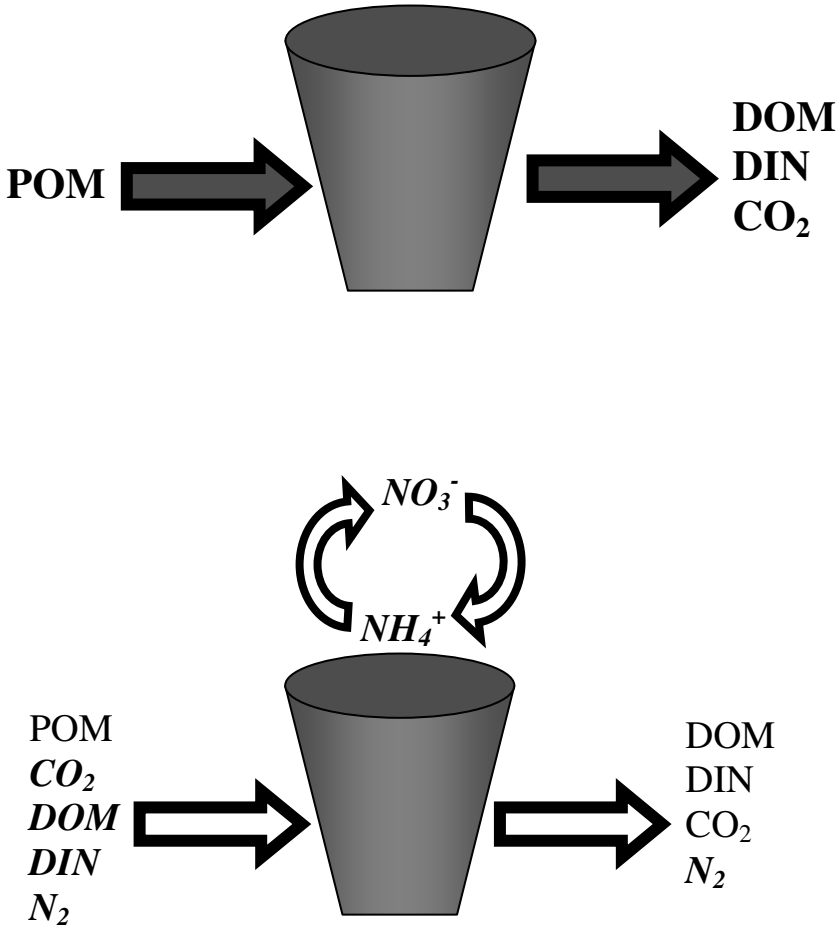

Figure 3.1. Expected fluxes of C and N in LMA sponges (top panel) and HMA sponges (bottom panel). Fluxes controlled by microbial community are in italics.

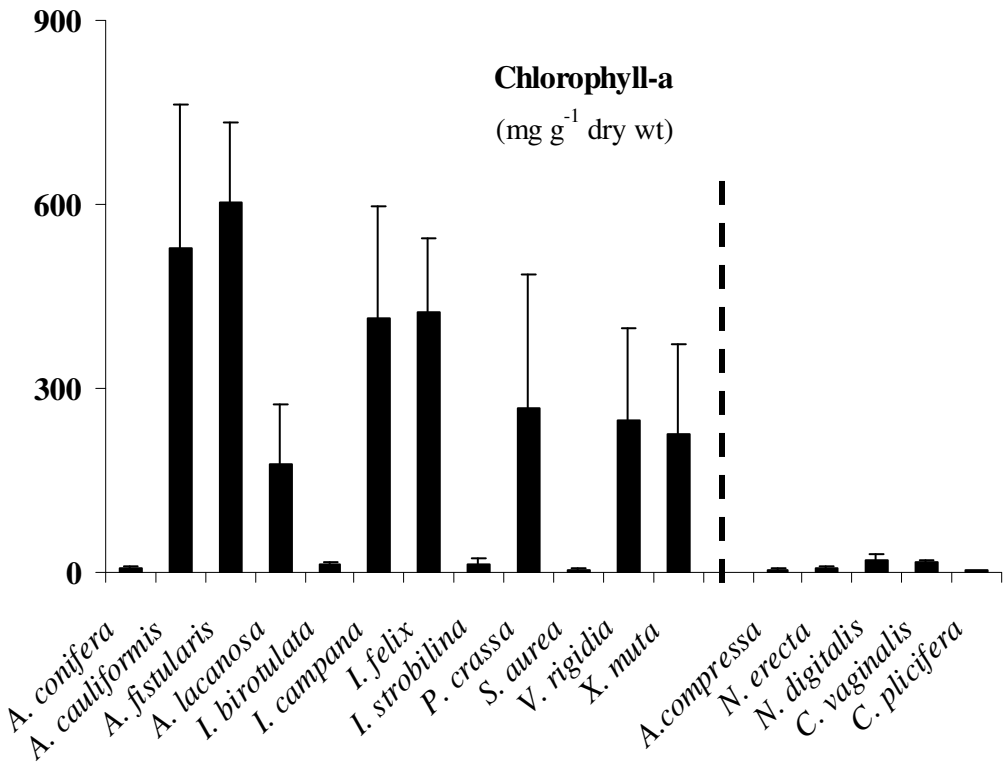

Figure 3.2. Chlorophyll-a concentrations in 16 speci es of sponge from Conch Reef. The dashed line separates HMA species from LMA species. Error bars are standard deviation.

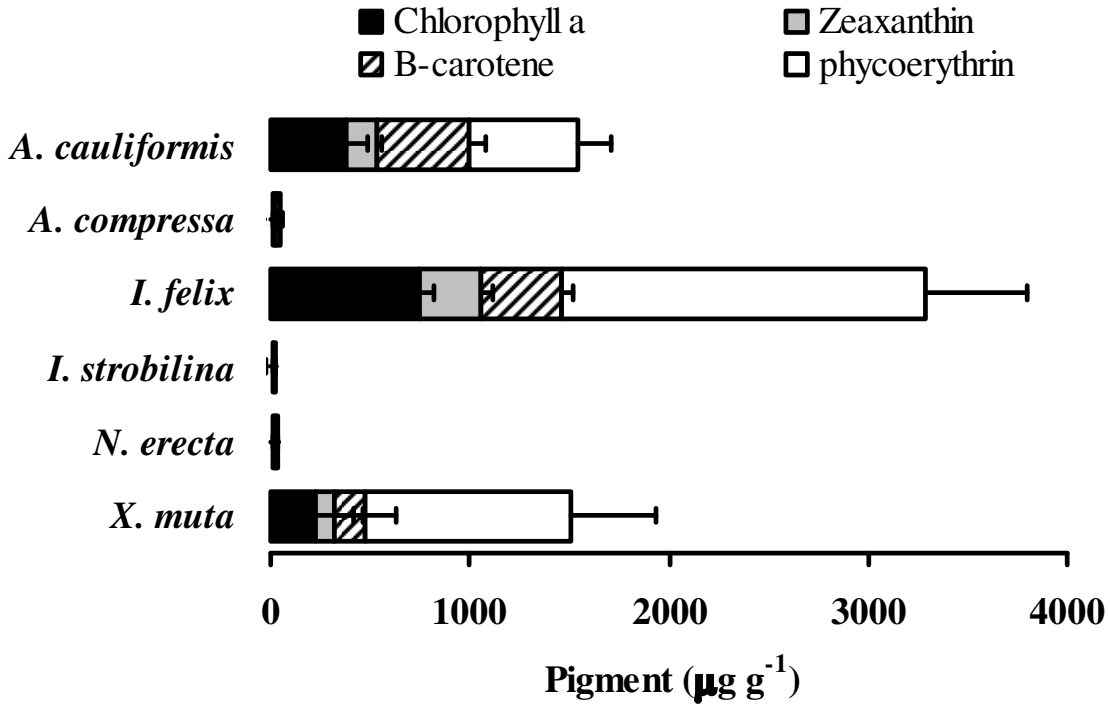

Figure 3.3. Pigment concentrations in sponge tissue. Chlorophyll-a, phycoerythrin, and carotenoid pigment concentrations (per g dry weight of sponge).

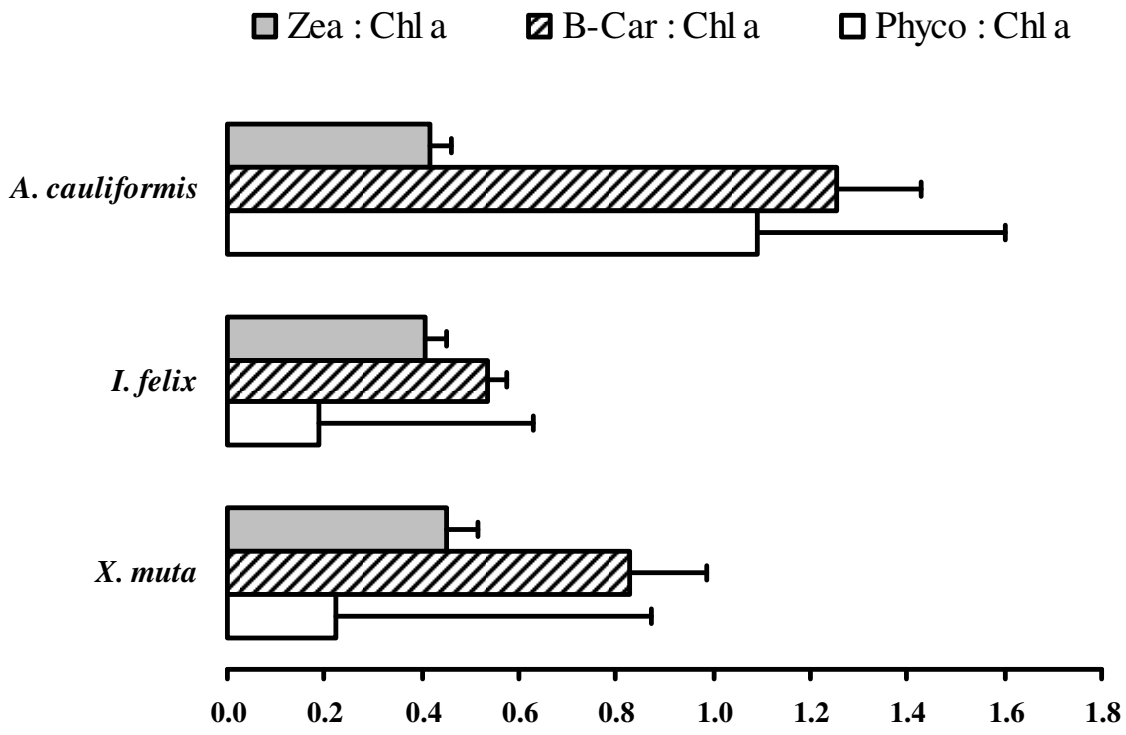

Figure 3.4. Pigment Ratios. Ratios of zeaxanthin, β-coartene, and phycoerythrin to chlorophyll-a in the outer 2 mm of sponge tissue. Error bars are standard deviation.

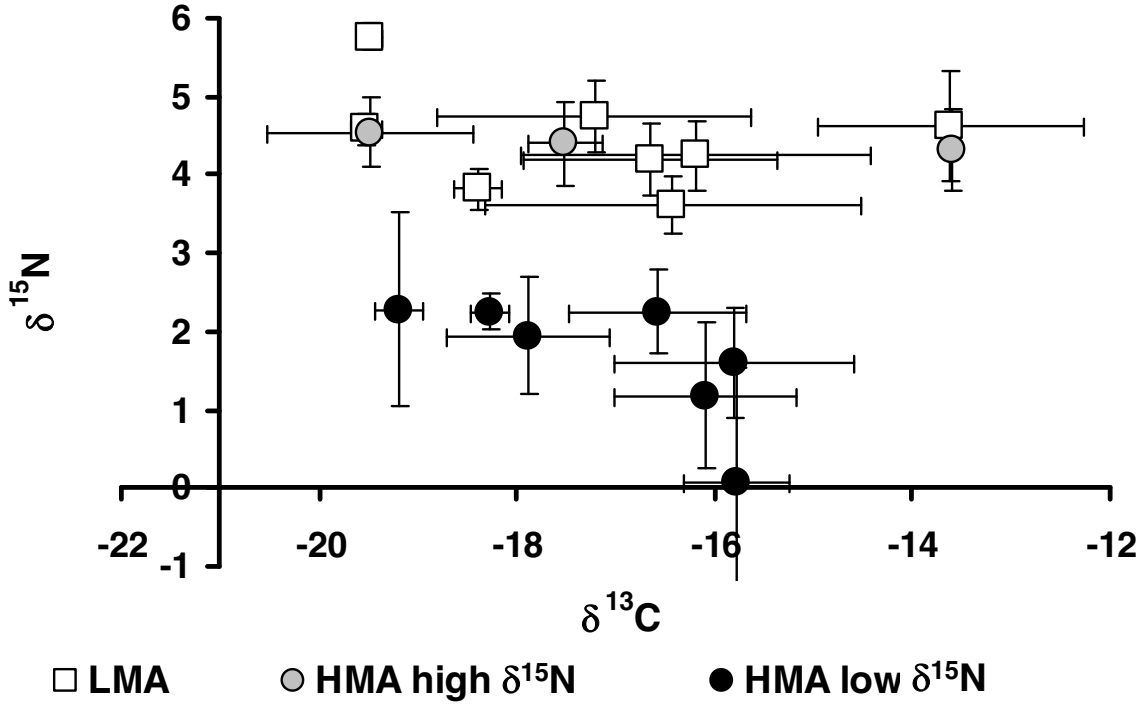

Figure 3.5. $\delta^{13}C$ and $\delta^{15}N$ values of HMA and LMA sponge species near Key Largo, FL. Error bars represent standard deviation.

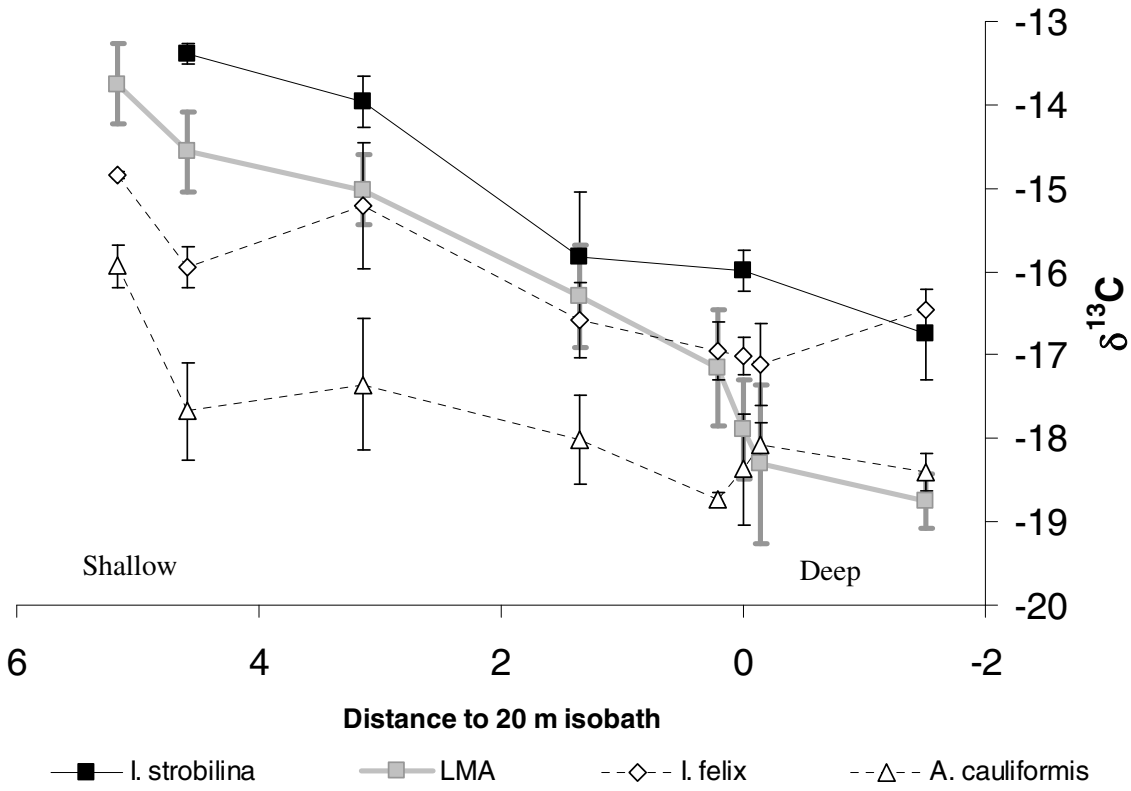

Figure 3.6. Three HMA species means and the LMA group mean $\delta^{13}C$ values along an on-shore to off-shore transect. Error bars represent standard deviation.

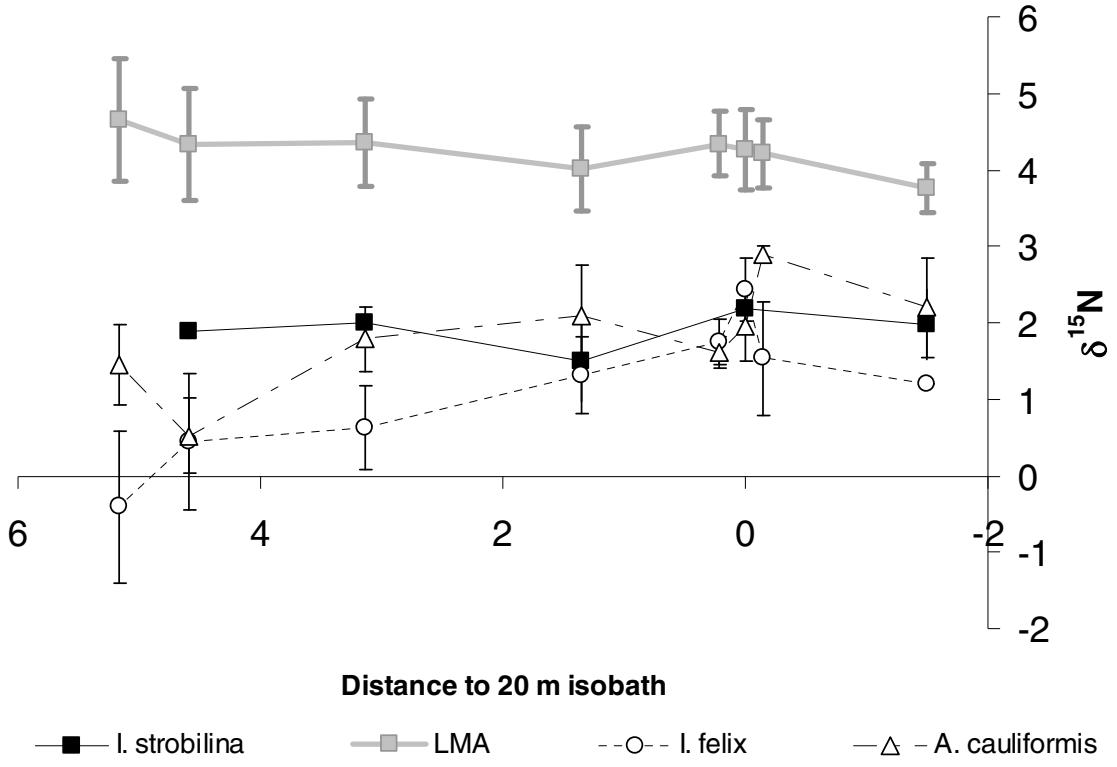

Figure 3.7. Mean $\delta^{15}N$ values of 3 HMA and LMA group mean along an on-shore to off-shore transect. Error bars represent standard deviation.

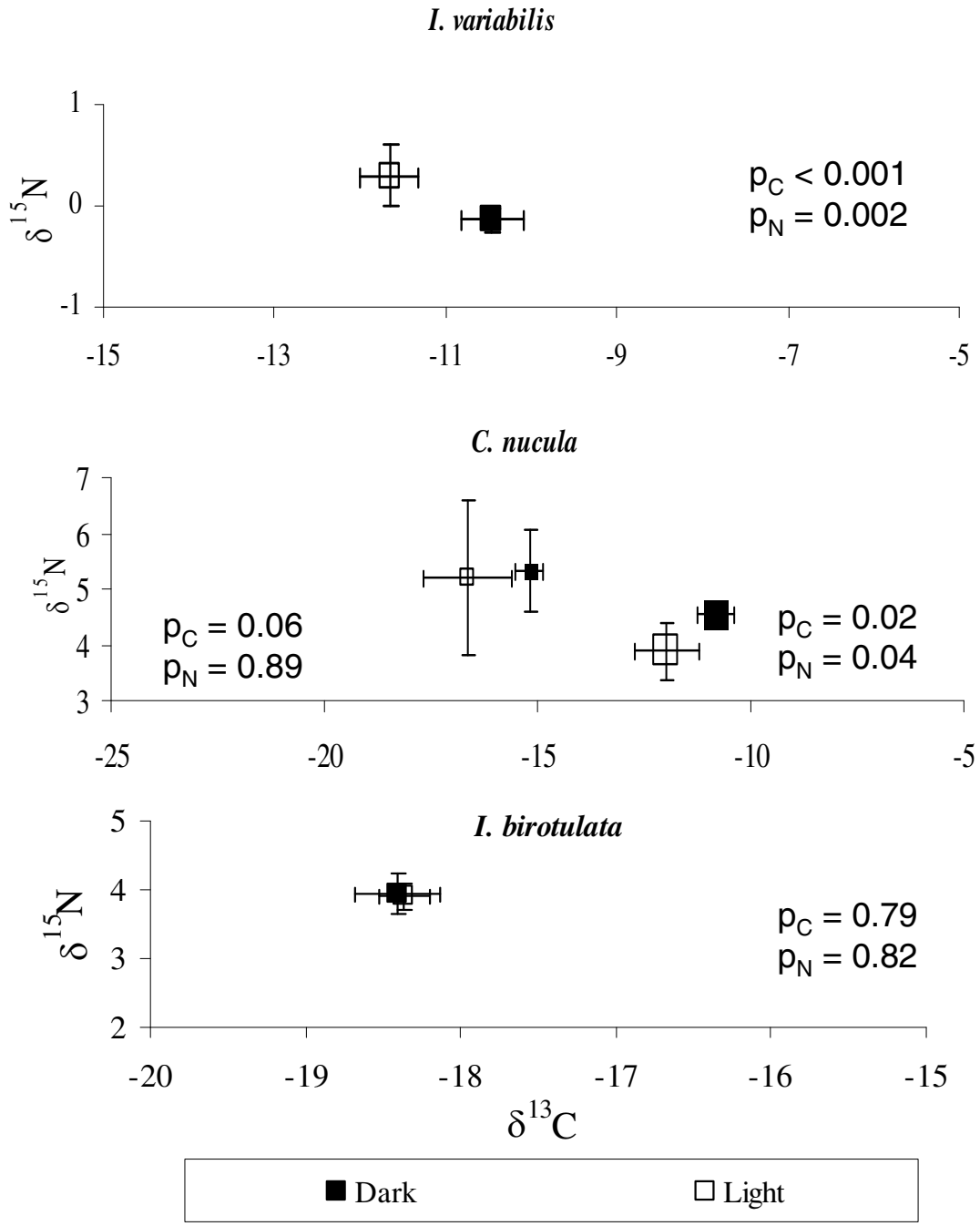

Figure 3.8. $\delta^{15}N$ and $\delta^{13}C$ values of bulk sponge tissue from individuals in shaded and well-lit locations. For *C. nucula*, small symbols represent Grouper Creek, and large symbols represent Cowpen Cut. Values for p_C and p_N represent p values for differences of $\delta^{13}C$ and $\delta^{15}N$ means, respectively. Error bars represent standard deviation.

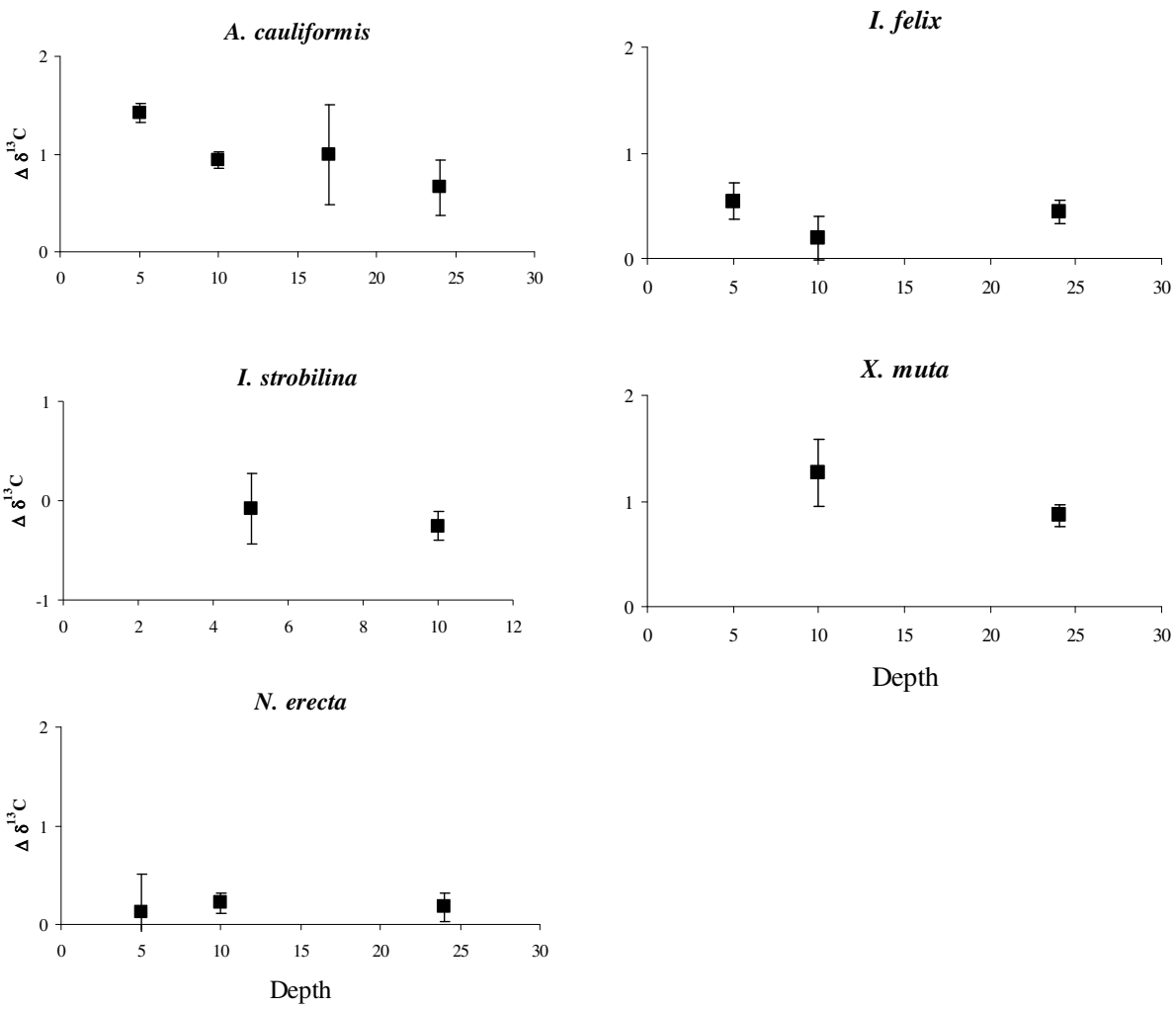

Figure 3.9. $\delta^{13}C$ values of **e**ctosome – $\delta^{13}C$ endosome ($\Delta\ \delta^{13}C$). Units are ‰, error bars are standard deviation.

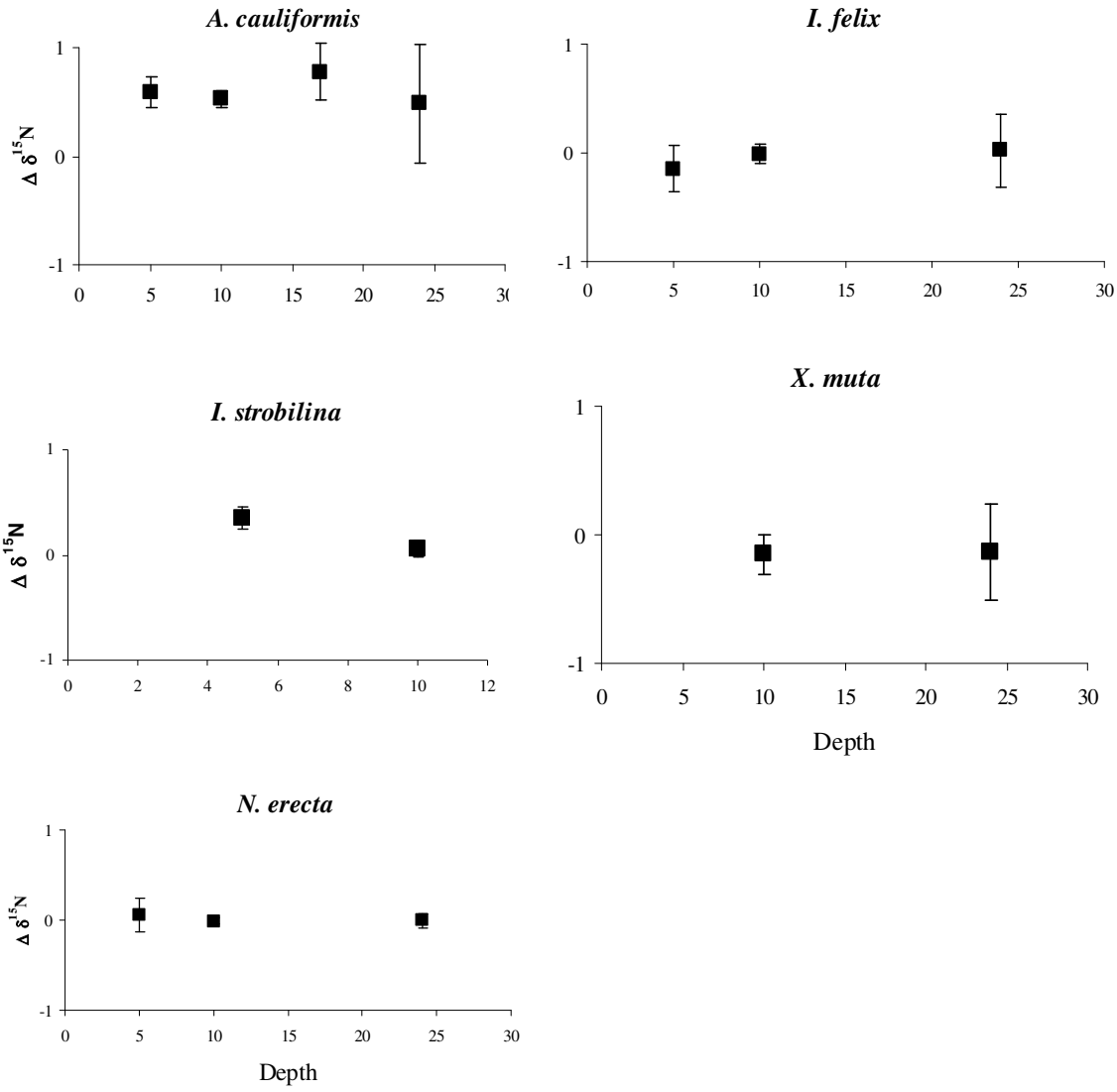

Figure 3.10. $\delta^{15}N$ values of ectosome – $\delta^{15}N$ of endosome ($\Delta\ \delta^{15}N$), units are ‰, error bars are standard deviation.

CHAPTER 4

In situ fluxes of dissolved inorganic nitrogen from the sponge community on Conch Reef, Key Largo, Florida

Predicting coral reef health in the face of climate change, disease, and eutrophication requires a comprehensive understanding of nutrient and organic matter cycling. Benthic reef organisms are known to alter suspended particulate organic matter and nutrient concentrations in water passing over the reef (Webb et al. 1975, Bak 1998, Yahel et al. 1998). Recent studies suggest that sponges can be a significant sink for POM (Ribes et al. 2005). Further, the removal of small particles and the increase in nitrate plus nitrite (NO_x^-) concentrations observed in reef waters (Van Duyl et al. 2002) are consistent with sponge feeding and nutrient excretion (Corredor et al. 1988, Pile 1997, Diaz and Ward 1997, Scheffers et al. 2004).

The ability of sponges to filter large volumes of water, up to 100,000 times their own volume every day (Weisz 2006), combined with their associated microbial communities (Hentschel et al. 2006, and references therein) influence their biogeochemical impact on coral reefs as well as other coastal environments where they are abundant. This large filtering capacity concentrates organic matter and delivers oxygen to the sponge, fueling fuels respiration rates up to 59 μmol O_2 (g dry weight)$^{-1}$ d^{-1} (Reiswig 1974, Wilkinson 1983) and

makes sponges a major factor in organic matter and nutrient cycling in coral reef ecosystems.

The few existing surveys of Caribbean reefs that report both size and abundance of sponges indicate that they outrank corals in terms of biomass (Diaz and Rutzler 2001 and references therein). Despite being a dominant feature of reefs, sponges are vulnerable to environmental factors such as bleaching events, hurricanes, commercial harvesting, and algal blooms (Vicente 1990, Butler et al. 1995, Cropper and DiResta 1999). Data on long-term changes of sponge populations are limited in part because of the large number of species and the morphological variability among individuals of the same species. However, a recent 14 year study revealed a dramatic, steady decline in the abundance (42.6%) and diversity (51.3%) of Caribbean sponge populations (Wulff 2006). Given their potential to influence the biogeochemistry and the ecology of the reef, declining sponge populations could have significant consequences for coral reef health.

It has been previously suggested that rapid rates of organic matter remineralization by some sponges might make them an important source of dissolved inorganic nitrogen (DIN) for Caribbean reefs (Corredor et al. 1988, Diaz and Ward 1997). Furthermore, some "high microbial abundance" (HMA, sensu Hentschel et al. 2006) species host large, diverse communities of microorganisms that may also affect the magnitude and/or the speciation of the DIN flux (Diaz and Ward 1997). Previous studies quantified DIN flux from key species in the sponge community, however, they used incubation chambers (Corredor et al. 1988, Diaz and Ward 1997) that required removal of sponges from the reef substrate. Even when care is taken to avoid injury to the sponge, manipulation can change its pumping rate and possibly its metabolic rate (Reiswig 1975).

Here we present NH_4^+ and NO_x^- (nitrate plus nitrite) fluxes from 14 common species of sponge on Conch Reef (Key Largo, FL, USA) measured using a combination of incubation and *in situ* methods. The *in situ* method is a novel approach that requires no physical contact with the sponge. A net DIN flux (defined as NH_4^+ plus NO_x^-) from non-encrusting sponges at Conch Reef was achieved by combining species-specific DIN flux results with a detailed sponge biomass survey of Conch Reef. The results were utilized to compare the two methods and also the overall importance of sponges relative to other DIN sources reported in the literature.

Methods

Net DIN fluxes from sponges were measured using either incubation or *in situ* methods. For the *in situ* method, the flux of each individual sponge was calculated as the product of its pumping rate and ΔDIN, where ΔDIN is defined as the difference in DIN concentration between ambient water (water immediately adjacent to the sponge) and the excurrent water (water exiting the sponge osculum). The *in situ* method was used whenever the sponges had an osculum large enough to enable sampling excurrent water and to measure flow velocities. Sponges with small oscula (e.g. rope morphology) were measured using a laboratory incubation time course method. In this method, DIN flux was determined by measuring the increase in DIN over time using incubation experiments. To evaluate the new method, DIN fluxes were measured from *X. muta* and *A. conifera* using both methods and the results were compared.

Study sites

Water samples were collected from Conch Reef near the Aquarius habitat, (see Chapter 2 for locations). Our sampling took place in July and August 2005 along a ridge at approximately 15 m depth. As Conch Reef is in a marine sanctuary, sponges for incubation experiments were obtained from Three Sisters, a patch reef approximately 6.8 km east of Key Largo.

***In situ* sampling**

SCUBA divers collected paired samples of sponge excurrent water and ambient water (defined above) in triplicate. Samples were taken in acid-washed 60ml syringes fitted with a stopcock and a short length of narrow tubing. Fluorescein dye was used to confirm that the sponge was pumping before the samples were collected. Excurrent samples were taken slowly (~ 2 mL s^{-1}) in order to obtain only water from the excurrent plume, and the syringes were first flushed with sample before the final collection. The samples were kept on ice until brought back to the lab, where a 12 mL aliquot was immediately analyzed for ammonium, and the rest frozen (-20^{o}C) in acid-washed 50 mL centrifuge tubes for later analysis of nitrate plus nitrite.

Pumping rates were measured using the method of Weisz (2006). Briefly, SCUBA divers recorded the upward movement of fluorescein dye injected into the excurrent plume using underwater videography ($n \geq 20$). A weighted stand with a flexible arm was used to position a ruler behind the sponge for scale. Pulses of dye were also released across the diameter of the osculum to determine the geometry of the excurrent plume. The dimensions of the sponge individuals were recorded to calculate tissue volume.

Incubation experiments

SCUBA divers cut pieces of sponge from healthy adults (2-8 cm^3, depending on species), attached them to PVC plates with plastic cable ties, and then left them on the reef to recover. Whenever possible, small, intact individuals were used rather than cuttings. Sponges recovered on a time scale of days to weeks, depending on species. Only sponges with healthy appearances (i.e. no visible wounds or decay) were selected for the experiments. These sponges were incubated in 2-4 L HDPE containers of seawater (obtained from Three Sisters Reef) for 4-8 hrs, and sampled at 4-5 time points during the incubation. The water in the incubation chambers was aerated and stirred using an aquarium pump. The incubations were performed outdoors in the shade, and containers were submersed in a flowing seawater bath to maintain ambient temperature. Two aliquots of water were taken at each time point; one was analyzed immediately for ammonium, and the other was frozen at -20^oC in an acid-washed (10% HCl) 50 mL centrifuge tube for nitrate plus nitrite analysis approximately one month later. A subset of samples was analyzed for nitrite only. At the end of the experiment, the sponges were measured, dried, and weighed for determination of mass and volume.

Nutrient analysis

Ammonium was measured by fluorescence following the method of Holmes et al. (1999). Briefly, 3 mL of o-phthalaldehyde working reagent was added to 12 mL of sample in an acid-washed 15mL centrifuge tube. Samples were incubated in the dark for 2 hours, and then analyzed using a Turner Designs fluorometer, model TD-700, fitted with an ammonium optical kit. The limit of detection for ammonium was determined to be 0.2 μmol L^{-1} by repeated measurement of standards. Ammonium standards were made fresh each day, and

measured along with samples. Nitrate plus nitrite (NO_x^-) was measured using standard colorimetric methods on a QuickChem flow-through autoanalyzer (Strickland and Parsons, 1972). A subset of samples was run to evaluate the potential contribution of nitrite. The limit of detection for nitrate plus nitrite was determined to be 0.25 μmol L^{-1} by repeated measurement of standards, and triplicate samples had an average standard deviation of 0.17 μmol L^{-1} for both ammonium and nitrate.

Statistical treatment

For calculation of ΔDIN, values below the analytical limit of detection were replaced with the limit of detection. Outliers were identified as follows: for each species, ambient and excurrent values were grouped, and values that were more than 1.5 times the interquartile distance above the third quartile were discarded. The differences between the means of ambient (n=3) and excurrent (n=3) samples for each sponge individual calculated, and the pooled estimate of variance for the difference between the two means was used in combination with flow measurement standard deviation to calculate a flux and standard deviation by propagation of error. Standard deviation in the measurement of sponge volume was estimated to be 20% and this value was also incorporated into volume normalized flux calculations by propagation of error. Unless otherwise noted, uncertainties are expressed as standard deviation. For incubation experiments, least squares regression of concentration versus time was used to calculate the flux, which was then normalized for the sponge volume and the incubation chamber volume, and flux standard deviation is based on fluxes from replicate individuals of each species.

Sponge biomass survey

SCUBA divers used nylon rope to establish a 20 X 30 m grid on Conch Reef marked at 1 m intervals. Divers then recorded the species, grid coordinates, and dimensions of every sponge in the 600 m^2 area. Branching sponges of the same species that were connected were counted as a single individual. The area covered by encrusting sponges was recorded, but DIN flux was not measured from any encrusting sponges, so the calculated community flux does not include their contribution.

Results and Discussion

In the 600 m^2 grid on Conch Reef, we measured 3971 sponges (3558 non-encrusting and 413 encrusting) comprised of 26 different species, and found an average of 6.5 sponges m^{-2} (Table 4.1). The volume of non-encrusting sponges averaged 3.60 L m^{-2} and coverage of encrusting sponges averaged 80 cm^2 m^{-2} of reef substrate. *Amphimedon compressa* had the highest number of individuals (1 m^{-2}). *Xestospongia muta*, a massive barrel sponge, dominated in terms of biomass, representing 65% of total sponge biomass (Table 4.1). Because of the large number of species present, it was not possible to measure DIN flux from the entire sponge community. However, by targeting the most abundant sponges, the 14 species measured comprised 85% of the non-encrusting sponge biomass in the survey area. Although species composition was different from another study of Caribbean reefs (Wulff 2006), the total volume of sponge biomass was similar.

Pumping rates and ΔDIN measurements were used to calculate in situ fluxes for 22 sponge individuals comprising 6 species (Table 4.2). Incubation experiments were used to

measure fluxes from 24 individuals comprising 8 species (Figure 1a,b). The calculated DIN flux from the 14 measured species of sponge was found to be 550 $\pm$ 120 μmol $m^{-2}h^{-1}$, of which 57 $\pm$ 73 μmol $m^{-2}h^{-1}$ was ammonium and 490 $\pm$ 91 μmol $m^{-2}h^{-1}$ was nitrate plus nitrite (Table 4.3). Nitrite was measured in a subset of samples from two species (*S. aurea* and *X. muta*) and the concentration was found to be negligible (data not shown). DIN fluxes among the species (normalized for sponge volume) averaged 210 $\pm$ 90 μmol L^{-1} h^{-1} (Table 4.3).

The DIN fluxes were not measured for 12 species representing 15% of sponge biomass in the grid. If we assume that these remaining sponges have DIN fluxes similar to the volume-weighted species average for the other 14 species (210 $\pm$ 90 μmol L^{-1} h^{-1}), we can estimate that these species are releasing an additional 110 $\pm$ 50 μmol m^{-2} h^{-1}. The sum of the measured and estimated fluxes therefore provides an estimate of DIN flux from the entire non-encrusting sponge community of approximately 660 $\pm$ 130 μmol m^{-2} h^{-1}. Most of this total flux is in the form of nitrate because *X. muta* (which releases primarily nitrate) dominates the community in terms of biomass and DIN flux (Table 4.1, Figure 4.3). As previously stated, we did not include flux measurements from encrusting sponges in our study. Previous work has shown that encrusting sponges can also be a large source of DIN to the water column when their biomass is high (Diaz and Ward 1997). In our biomass survey, encrusting sponges covered only 0.8% of the substrate, however this could be an underestimate due to the difficulty in measuring cryptic encrusting organisms. Therefore, the estimated community total flux of 660 $\pm$ 130 μmol m^{-2} h^{-1} could be considered a minimum community DIN flux.

To our knowledge, this is the first study of DIN flux from sponges measured *in situ* with no manipulation of the sponge. We were able to employ the *in situ* method for 6 of the

14 species measured. This translates into 76% of sponge biomass in the survey grid, and 90% of biomass for which we have flux data. There were some differences in the flux rates obtained from the method comparison using *X. muta* and *A. conifera*. For *X. muta*, the incubation method produced nitrate fluxes that were lower than the in situ method (Figure 4.4); however these differences were not statistically significant for either NO_x^- (p=0.07), NH_4^+ (p=0.22), or total DIN (p=0.09). *X. muta* was the largest sponge incubated and so may have depleted the food supply in the incubation chamber, although there may be a time lag between reduction in food supply and reduction in DIN release. The nitrate time course data for *X. muta* are linear (Figure 3, R^2= 0.97) and show no signs of leveling off over time. Nevertheless, food limitation could be the reason for the lower flux using the incubation method. For *A. conifera,* the two methods produced similar fluxes for total DIN (p=0.41), but more NH_4^+ (p=0.01) and less NO_x^- (p=0.03) was released with the incubation method compared to the in situ method (Figure 2). This suggests that the experimental manipulation may have affected the nitrifying community hosted by this sponge (Corredor et al. 1988, Diaz and Ward 1997, Southwell, M. W., et al. in prep). Unlike *X. muta*, it was not possible to find intact individuals of *A. conifera* small enough for incubation, and cutting individuals may have isolated parts of the sponge with fewer nitrifiers, or disrupted the nitrifiers that were present, despite the time allowed for recovery. For these reasons, results from the *in situ*, rather than the incubation method, were used in calculating total community benthic flux. However, despite the differences, the total DIN flux from these two methods were not statistically different, which is remarkable given the difference in size necessary for the two methods and the relatively crude conditions of the incubation experiments. This suggests that previous estimates of DIN flux that solely employed incubation methods (Corredor et al.

1988, Diaz and Ward 1997, Pile 1997) are valid when care is taken in the experimental conditions and manipulation of the sponges.

The *in situ* flux measurements were performed in a 5-10 minute interval and so represent a "snapshot" of sponge DIN flux. Incubation experiments provided information over a longer time frame however the experiments lasted 8 hours or less. Therefore, extrapolating to daily (or longer) flux rates substantially increases the uncertainty. Sponges are known to slow their pumping rates periodically (Reiswig 1971, Weisz 2006); however, it is not known how this behavior affects the overall DIN flux during this time, because the excurrent water may become more concentrated as the flow subsides. Conversely, the metabolic rate of the sponge (and possibly the associated microbes) may also decrease during this time of reduced pumping activity, reducing the production of metabolic waste. In addition to reductions in pumping activity due to behavioral changes, Reiswig (1971) also cited temperature and physical disturbance (e.g. storms) as causative factors of depressed pumping rates.

All sponges in this study known to be HMA (high microbial abundance) sponges released significant amounts of NO_x^-. The phenomenon of sponge-hosted nitrification has previously been documented (Corredor et al. 1988, Diaz and Ward 1997) and will be further discussed in the next chapter. For the purposes of comparing species means of total DIN flux, I have converted the volume-normalized flux rates into mass-normalized flux rates using density data from Weisz 2006. This is because LMA sponges generally have lower density, and so would be expected to release less DIN than an HMA sponge of equal volume and equal metabolic activity. Figure 4.5 shows that HMA sponges generally release less DIN compared to LMA sponges, except for the genus *Ircinia*. With *Ircinia* species included in

the analysis, none of the differences between species type are significant. However, when *Ircinia* is excluded from the comparison, HMA sponges (Chapter 3) release significantly less DIN compared to LMA species ($p = 0.04$). The DIN fluxes of HMA sponges with resident microalgae were not significantly different from those that lack them ($p = 0.88$). The lower DIN flux rate of HMA sponges (excluding *Ircinia*s) is consistent with the partial retention of metabolic waste DIN by the microbial community. The lack of difference between species with and without microalgal associates then suggests that heterotrophic microorganisms could be participating in DIN uptake. However, *Ircinia* sponges are also classified as HMA, and it is unclear why this genus should release more DIN than LMA species.

Approximately 72% of the measured benthic DIN flux is from *X. muta* (Table 4.3), an HMA sponge that hosts abundant cyanobacteria in its surface tissues (Chapter 3). If cyanobacteria are assimilating a portion of metabolic waste DIN, it could be hypothesized that DIN release is reduced during daylight hours due to uptake of DIN for photosynthesis. To evaluate the consistency of the DIN flux, one *X. muta* individual was sampled intensively over the course of a day. The average ΔDIN was 0.61 ± 0.23 μmol L^{-1} and ranged from 0.36 to 1.04 μmol L^{-1}, and ΔDIN remained generally consistent over the sampling period (Figure 4.6). The data show no apparent correlation with time of day, suggesting that uptake of DIN by photoautotrophs is negligible, or that the uptake continues during dark hours. Together with the lack of difference between sponges with and without microalgal associates, this result suggests that phototrophy does not significantly affect the net flux of DIN from sponges. This result is surprising, given the expected nutrient demand from photosynthetic cells. Nevertheless, little is known about the effect of microbial communities on the net DIN

flux of sponges, and how this flux varies over time. These factors should be considered when extrapolating estimates of DIN release by sponges to daily (or longer) time scales.

Sponges could potentially act as a conduit for nutrients from the pelagic to the benthic system. Although they feed solely on planktonic and dissolved organic matter sources (Pile 1997, Yahel et al. 2003), the remineralized N that sponges release is available to both planktonic and benthic algae, likely resulting in a net transfer of nutrients from pelagic to benthic biomass. The sponge community DIN flux is more than 10 times rates reported in the literature for other benthic sources such as sediments and coral cavities (Table 4.4). Furthermore, the fluxes calculated for sediments and coral cavities assumed 100% coverage, and may therefore be overestimates of actual benthic flux. In contrast, the calculated fluxes for sponges (Corredor et al. 1988, Diaz and Ward 1997, and this study) are based on measured sponge abundance. This release of nutrients near the reef could have ecological consequences, as high nutrient availability may facilitate the growth of fleshy macroalgae (Littler and Littler 1984, Larned 1998), which have increased in abundance on Caribbean reefs (Done 1992). Lapointe (1997) has argued that DIN concentrations of 1 μmol L^{-1} or more are associated with rapid macroalgal overgrowth, and our data show that sponge excurrent water can be as high as 5 μmol L^{-1} (*I. strobilina*).

Coral reefs are capable of supporting high rates of primary productivity, despite low nutrient concentrations in the water column. Reported rates of benthic net primary production on various coral reef substrates show a wide range, from 8 to 417 mmol C m^{-2} d^{-1} (Table 4.5). This implies a nitrogen requirement of 1 to 63 mmol N m^{-2} d^{-1}, assuming a C:N ratio of 6.6 (Redfield 1958). The non-encrusting sponge community on Conch Reef releases approximately 16 mmol m^{-2} d^{-1}, and is therefore likely capable of supporting a significant

amount of the benthic primary productivity. Sponge DIN may be largely derived from the remineralization of POM, and in some sponges DOM (Yahel et al. 2003) and is therefore recycled rather than "new N". However, stable isotopic evidence suggests that N_2 fixation could be hosted in some HMA sponges (Chapter 2), which could also contribute to the observed flux. For example, members of the genus *Ircinia* in this study are all HMA sponges with low $\delta^{15}N$ values, and high mass-normalized fluxes of DIN. These characteristics are consistent with N_2 fixation by the microbial community (N_2 fixation is discussed further in Chapter 6).

High nutrient availability may affect coral reef health in ways other than influencing macroalgal abundance. The connection between inorganic nutrients and coral disease incidence is controversial (Szmant 2002, Kline et al. 2006), however there is evidence that elevated inorganic nutrient levels increase the virulence of coral pathogens (Bruno et al. 2003) and decrease coral fecundity (Koop et al. 2001). Further, higher rates of primary productivity generated by inorganic nutrient fluxes could produce more dissolved organic matter (Anderson and Zeutche 1970), which as been shown to be a potential factor in coral disease (Kline et al. 2006). Thus, DIN release by sponges could have multiple consequences for reef health and ecology.

Conclusions

Our *in situ* measurements of DIN fluxes from sponges on Conch Reef, Key Largo, USA, reveal that sponges are a large source of DIN and represent a significant pathway for recycling of organic matter on Caribbean reefs, and presumably other coral reefs where

sponges are abundant. The Conch Reef sponge population was dominated (in terms of volume) by sponges hosting nitrification, and so most of the DIN flux was in the form of nitrate. Although the incubation methodology appeared to affect the speciation of DIN released from one species, the overall DIN flux measurements made using the *in situ* method were not statistically different from those obtained using the incubation method. Therefore this study supports previous findings of the high magnitude of sponge DIN fluxes, and also provides a viable alternative method for measuring DIN flux that is non-destructive and reflects real environmental conditions. I believe that the *in situ* method is preferable to the incubation method for the following reasons: 1) environmental conditions that may affect sponge behavior and biogeochemical processes (e.g. light, temperature, etc.) are difficult to replicate in the laboratory, 2) any manipulation of the sponge has the potential to alter pumping and metabolic rates (Reiswig 1971), and 3) large sponges (e.g. *X. muta*) cannot be measured using incubation methodology, yet they likely have a proportionally greater impact on DIN fluxes compared to small sponges.

Changes in sponge biomass and speciation could affect POM concentrations, nutrient availability and benthic-pelagic coupling significantly throughout the coastal zone. On Conch Reef, the DIN flux was dominated by *X. muta*, a massive barrel sponge that appears to be partially vulnerable to bleaching events (Vicente 1990). Further, the response of sponges to environmental factors such as climate change and eutrophication is still largely unknown. Compared to corals, there are relatively few monitoring programs that provide information on sponge abundance, diversity, and health. Given the potential impact of these animals on N cycling, this lack of information could weaken our understanding of reef ecology and limit our ability to successfully maintain healthy coral reefs.

Tables

	Volume L m^{-2}	**Abundance** Ind. m^{-2}
Total		6.50
Encrusting		0.69
Non-encrusting	3.60	5.91
Agelas clathrodes	0.01	0.03
Agelas conifera	0.23	0.24
Agelas wiedenmeyeri	0.06	0.59
Amphimedon compressa	0.10	1.01
Aplysina archeri	0.01	0.04
Aplysina cauliformis	0.03	0.79
Aplysina fistularis	0.00	0.01
Aplysina fulva	0.02	0.07
Aplysina lacunosa	0.06	0.21
Callyspongia plicifera	0.02	0.09
Callyspongia vaginalis	0.13	0.35
Geodia sp.	0.02	0.01
Iotrochota birotulata	0.00	0.09
Ircinia felix	0.04	0.18
Ircinia strobilina	0.08	0.19
Mycale laxissima	0.00	0.02
Niphates digitalis	0.05	0.54
Niphates erecta	0.02	0.62
Pseudoceratina crassa	0.33	0.47
Smenospongia aurea	0.02	0.02
Verongia gigantea	0.03	0.01
Xestospongia muta	2.33	0.20
Unknown	0.01	0.01

Table 4.1. Results of biomass survey from 600 m^2 area of Conch Reef.

Species	ΔDIN (μmol L^{-1})				Flow Rate ($L\ h^{-1}$)		Sponge Vol. (L)	Flux (μmol $h^{-1}\ L^{-1}$)			
	NH_4^+		NO_x^-					NH_4^+		NO_x^-	
	Mean	SD	Mean	SD	Mean	SD		Mean	SD	Mean	SD
A. conifera	-0.10	0.03	0.78	0.07	420	58	1.6	-25	8	210	53
A. conifera	-0.11	0.11	0.51	0.12	280	38	1.1	-29	29	130	46
A. conifera	-0.23	0.23	0.42	0.07	670	63	1.7	-94	90	160	45
A. archeri	0.67	0.50	0.98	0.11	71	12	0.6	87	68	130	36
A. archeri	0.13	0.43	0.61	0.14	54	17	0.4	17	55	79	35
A. archeri	0.10	0.19	0.58	0.14	88	39	0.5	16	32	94	52
A. lacunosa	-0.29	0.65	1.78	0.20	22	7	0.4	-17	27	110	41
A. lacunosa	0.11	0.43	0.54	0.22	88	6	0.4	23	95	120	55
A. lacunosa	0.45	0.50	1.09	0.38	100	26	0.9	52	60	130	60
I. strobilina	0.06	0.08	0.54	0.13	670	110	1.3	28	43	270	93
I. strobilina	-0.01	0.06	0.36	0.12	2300	630	3.7	-7.5	27	220	100
I. strobilina	-0.05	0.04	0.69	0.12	630	100	1.2	-28	25	360	110
I. strobilina	0.18	0.12	1.26	0.13	920	250	2.0	81	58	610	210
I. strobilina[*]	1.03	0.11	2.69	0.22	1300	220	2.5	520	150	1400	380
N. digitalis	0.27	0.06	ND	ND	670	460	0.6	320	240	ND	ND
N. digitalis	0.17	0.07	ND	ND	580	370	0.5	210	160	ND	ND
N. digitalis	0.20	0.03	ND	ND	370	240	0.3	240	170	ND	ND
X. muta	-0.06	0.27	0.41	0.38	4000	150	16	-14	63	100	96
X. muta	0.03	0.32	0.81	0.20	13000	71	45	10	93	240	76
X. muta	-0.11	0.17	0.74	0.15	24000	120	109	-24	37	170	47
X. muta	0.09	0.27	0.62	0.37	18000	130	49	33	98	220	140
X. muta	-0.19	0.23	1.40	0.15	1900	280	24	-15	17	110	30

Table 4.2. ΔDIN, flow rate, volume and volume-normalized flux for individuals measured using the in situ method. SD = standard deviation; ND = Not determined; * Outlier not used in species mean or benthic flux calculation.

	Method	*N*	NH_4^+ μmol h^{-1} L^{-1}		NO_x^- μmol h^{-1} L^{-1}		NH_4^+ μmol h^{-1} m^{-2}		NO_x^- μmol h^{-1} m^{-2}	
			average	SD	average	SD	average	SD	average	
Agelas conifera	*In situ*	3	-49	32	170	28	-11	7	38	6
Aplysina archeri	*In situ*	3	40	31	100	23	0	0	1	0
Aplysina lacunosa	*In situ*	3	19	39	120	30	1	2	7	2
Ircinia strobilina	*In situ*	4	18	27	370	91	1	2	30	7
Niphates digitalis	*In situ*	3	260	110	ND	ND	14	6	ND	ND
Xestospongia muta	*In situ*	5	-2	31	170	39	-5	72	396	91
Aplysina cauliformis	Incubation	3	44	20	160	70	1	1	4	2
Aplysina fistularis	Incubation	3	68	22	24	53	0	0	0	0
Amphimedon compressa	Incubation	3	150	39	0	0	15	4	0	0
Callyspongia vaginalis	Incubation	3	290	64	2	1	35	8	0	0
Ircinia campana	Incubation	3	220	81	86	36	0	0	0	0
Ircinia felix	Incubation	3	41	16	270	63	1	1	10	2
Niphates erecta	Incubation	3	140	32	5	1	3	1	0	0
Smenospongia aurea	Incubation	3	5	2	230	32	0	0	4	1
Total							57	73	490	91

Table 4.3. Average DIN flux from 14 species. SD = standard deviation.

Substrate	**Flux** **(mmol m^{-2} d^{-1})**	**Location**	**Reference**
Coral Sediment	1.68	Great Barrier Reef	Capone et al. 1992
Coral Cavities	1.41	Caribbean	Scheffers et al. 2004
Sediment	0.613	Hawaii	Stimson and Larned 2000
Sponge (P. zeai)	5.8-10.9	Caribbean	Diaz and Ward 1997
Non-encrusting sponge community	**16 ± 3.1**	**Caribbean**	**This Study**

Table 4.4. Benthic fluxes of DIN on coral reefs from the literature. Uncertainties are standard deviation.

Substrate	Net primary production (mmolC m^{-2} d^{-1})	Reference
Sands	39	Uthicke and Klumpp 1998
Coralline algae	75 - 417	Chisolm 2003
Biofilms	31	Lugomela et al. 2005
Macroalgae	8 $\pm$ 5	Gattuso et al. 1997

Table 4.5. Benthic primary productivity rates from the literature. Uncertainties are standard deviation.

Figures

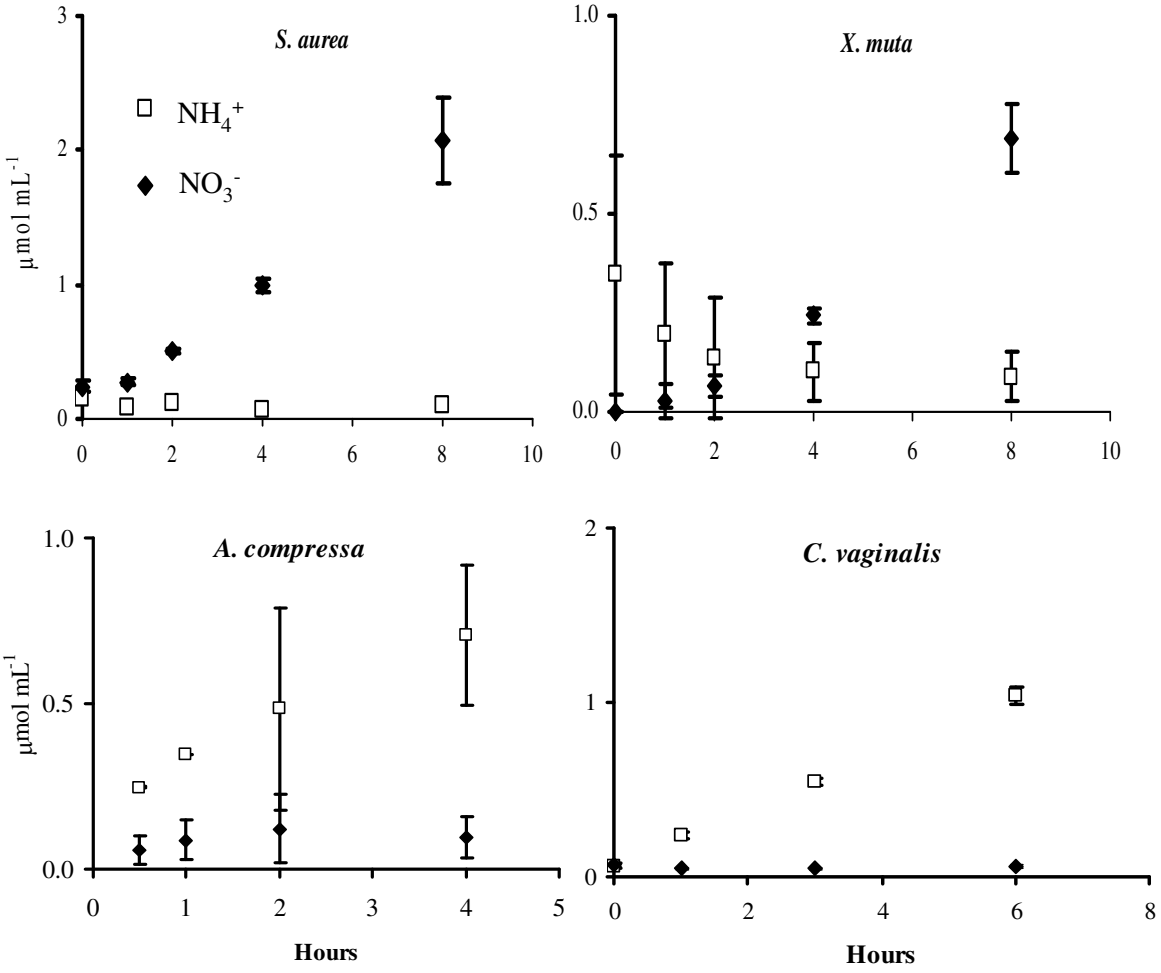

Figure 4.1a. Incubation experiments. Concentrations are normalized for incubation volume and sponge volume, error bars are standard deviation.

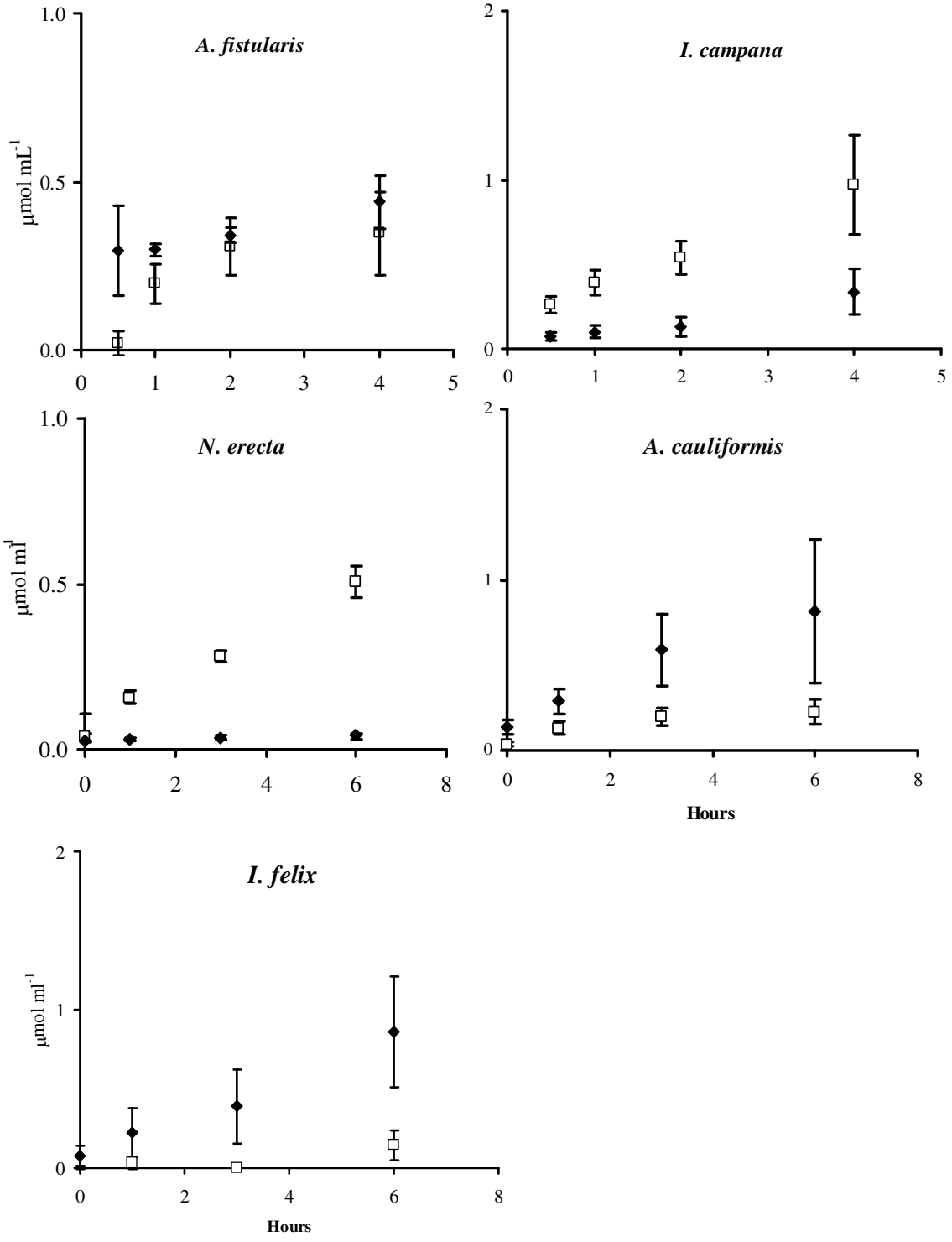

Figure 4.1b. Incubation experiments. Concentrations are normalized for incubation volume and sponge volume, error bars are standard deviation.

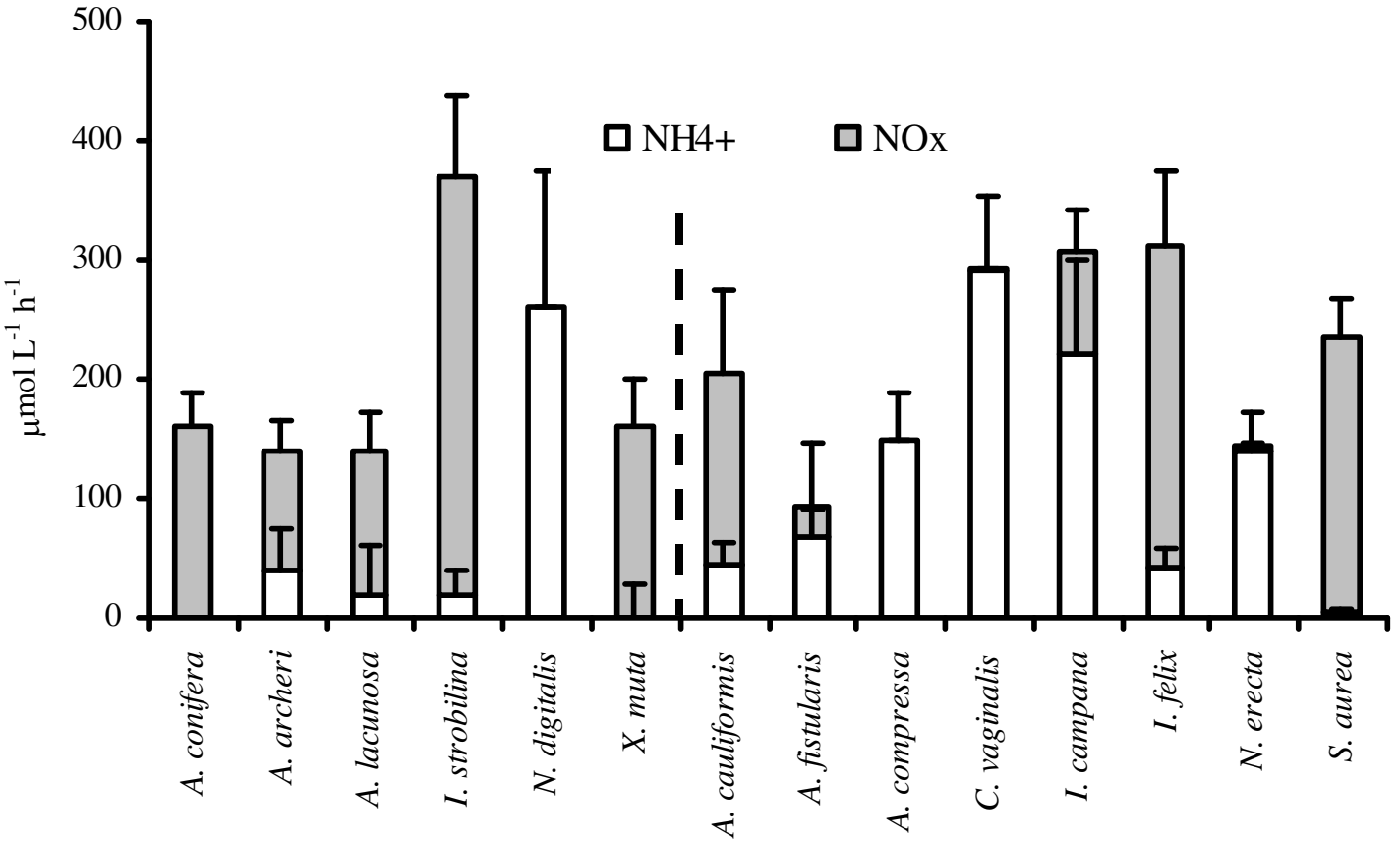

Figure 4.2. Volume-normalized DIN fluxes for 14 measured species on Conch Reef. Species to the left of the dashed line were measured using the *in situ* method, and species to the right of the line were measured using the incubation method. Error bars are standard deviation.

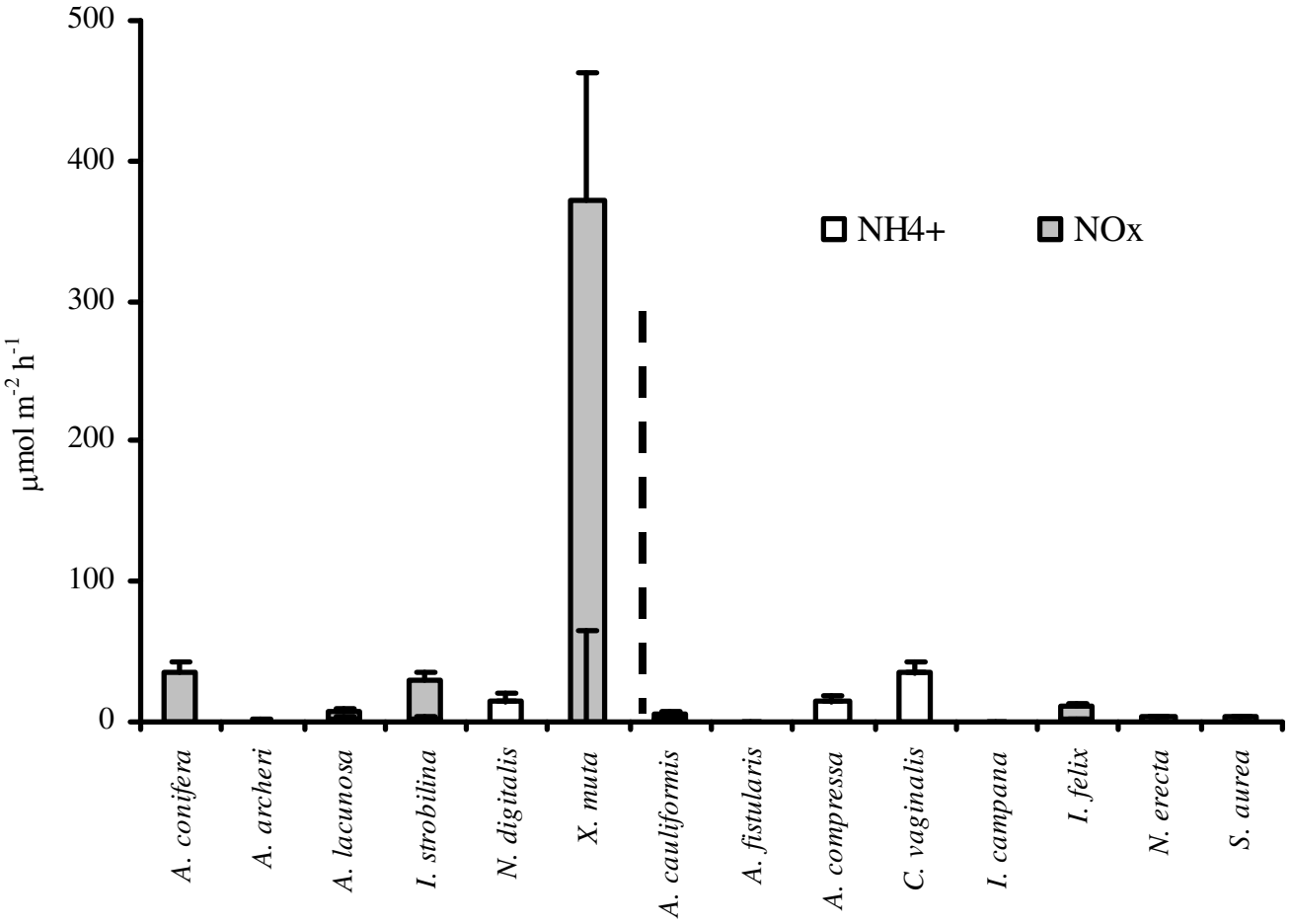

Figure 4.3. Benthic DIN fluxes for 14 measured species on Conch Reef. Species to the left of the dashed line were measured using the *in situ* method, and species to the right of the line were measured using the incubation method. Uncertainties are standard deviation.

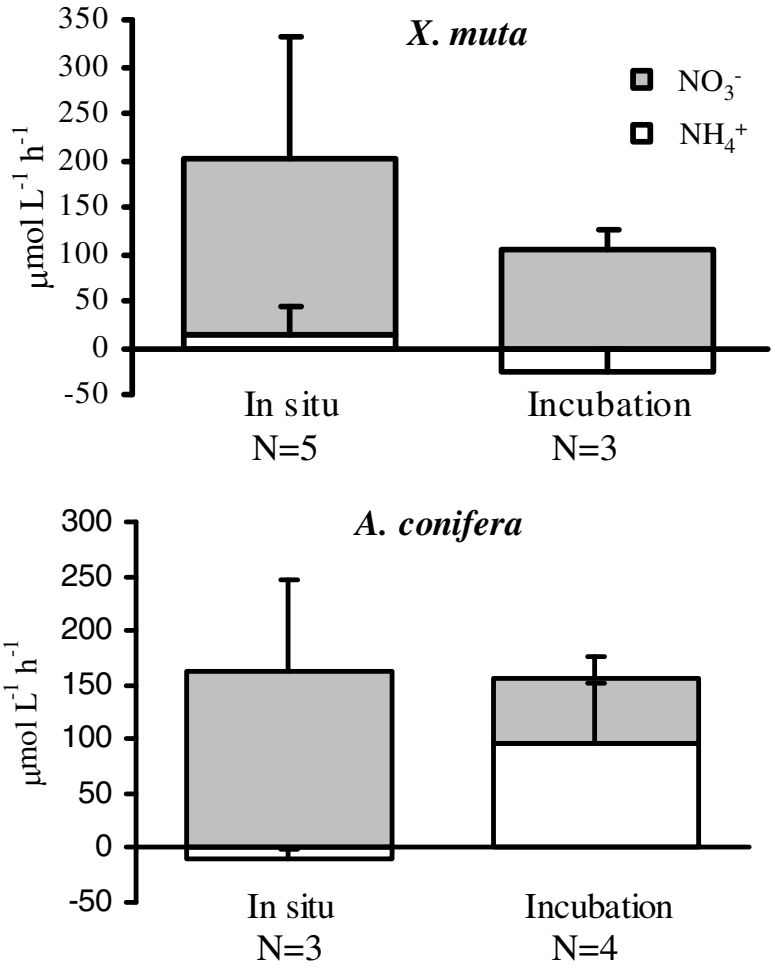

Figure 4.4. Comparison of results from *in situ* and incubation methods. Error bars are standard deviation.

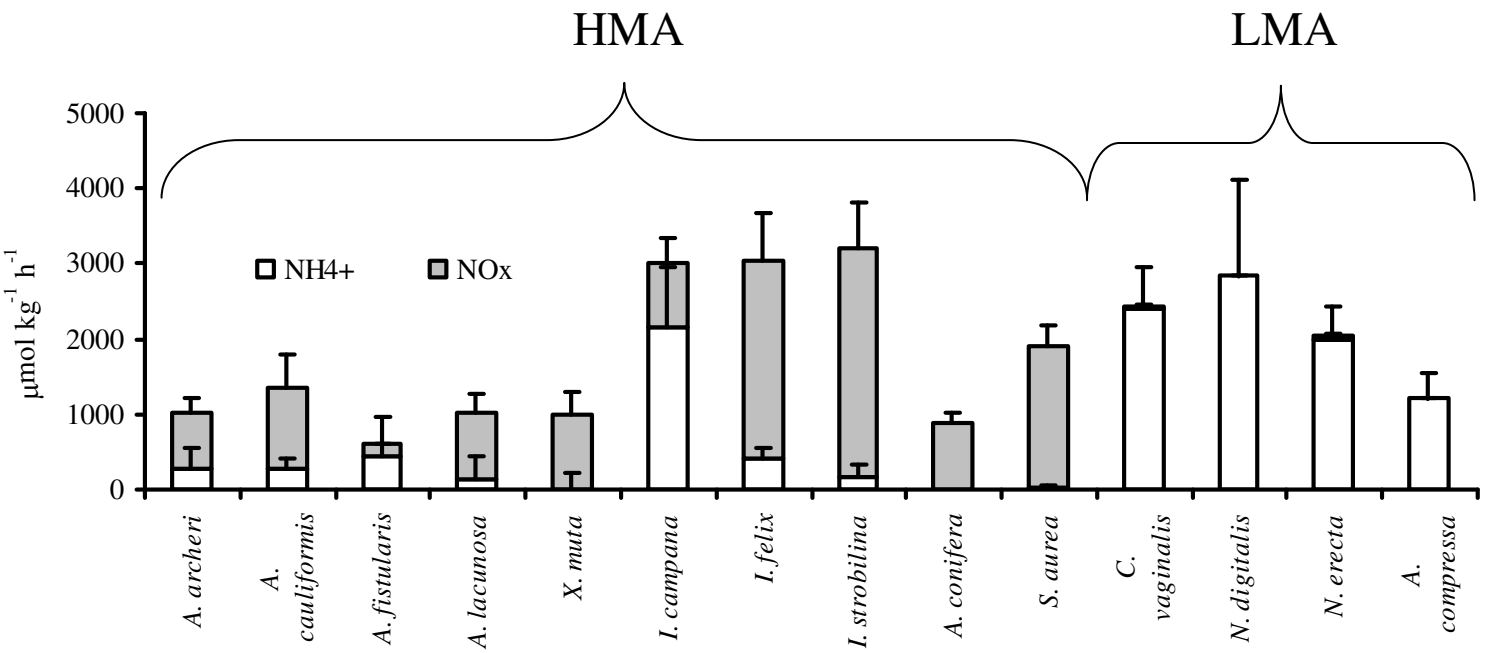

Figure 4.5. Mass-normalized DIN fluxes for 14 species on Conch Reef. Error bars are standard deviation.

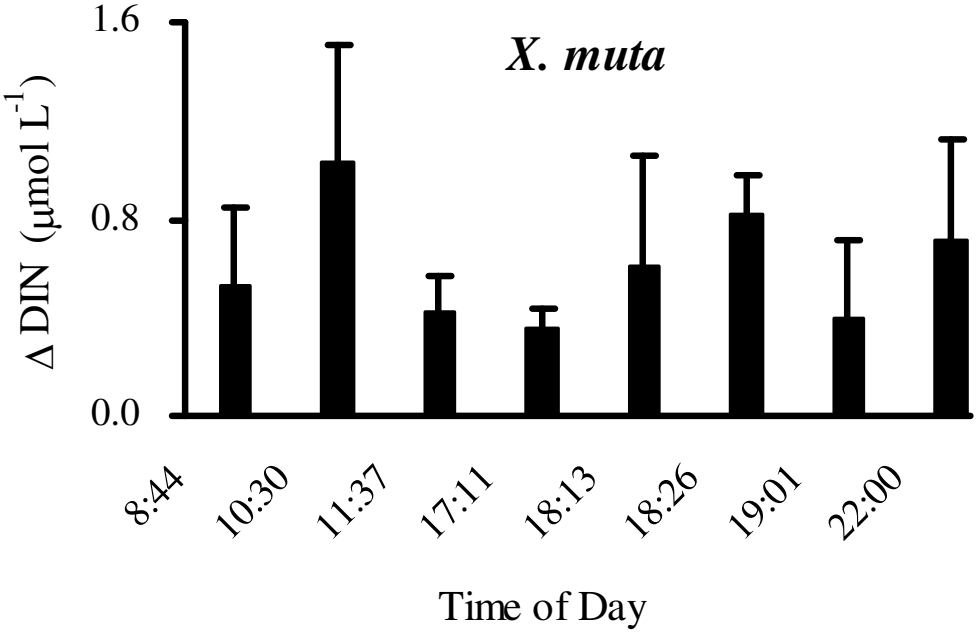

Figure 4.6. Time series of nutrients released by one individual *X. muta* sampled over the course of one day. Error bars are standard deviation.

CHAPTER 5

Nitrification controls on fluxes and isotopic composition of nitrate from Florida Keys sponges

Nitrification rates in sponges reported to date (Corredor et al. 1988, Diaz and Ward 1997, Pile 1996, Chapter 3) exceed those measured in other marine benthic systems such as microbial mats (Bonin and Michotey 2006), coral reef sediments (Capone et al. 1992), and continental shelf sediments (Hopkinson et al. 2001); yet surprisingly, little is known about this phenomenon at a mechanistic level. Ammonium oxidation can be performed by beta- or gamma- proteobacteria (Costa et al. 2006 and references therein), or by Marine Group 1 crenarchaeota (Konneke et al. 2005, Hallam et al. 2006). Although they had escaped detection until recently, ammonium-oxidizing archaea (AOA) have been shown to comprise a large portion of deep-water bacterioplankton (Karner et al. 2001) and could be responsible for much ammonium oxidized in the ocean (Francis et al. 2005). Neither bacteria nor archaea can oxidize ammonium completely; however, either of these groups can couple with bacterial nitrifiers to oxidize the resulting nitrite to nitrate (Costa et al. 2006).

Both bacterial and archaeal ammonium oxidizers have been detected in marine sponges (Preston et al. 1996, Hallam et al. 2006, Hentschel et al. 2006), along with a wide variety of other microbial organisms. It is unclear whether AOA or bacteria (or both) are responsible for the impressive rates of ammonium oxidation observed in sponges, and how this process may be connected to other concurrent microbially-mediated processes in sponges. Microbial communities known to live in sponges perform a diverse array of aerobic and anaerobic processes, including photosynthesis (Wilkinson 1983, Rutzler 1990), sulfate

reduction (Hoffman et al. 2005), methane oxidation (Vacelet 1996), nitrification (Corredor et al 1988, Diaz and Ward, 1997) and possibly N_2 fixation (Wilkinson and Fay 1979, Shieh and Lin. 1994, Wilkinson 1999). Given the density of sponge populations in some regions (e.g. Caribbean (2.1-3.6 L m^{-2}; Wulff 2006, Chapter 4), these sponge-hosted microbial processes may have an important impact on the overall biogeochemical cycling of carbon and nutrient elements in coral reef ecosystems.

The existence of sponge-hosted nitrification has been empirically proven for almost 20 years (Corredor et al. 1988); however, to my knowledge, no definitive experiments have been done to assess its importance among sponge species occurring in coral reef ecosystems, or to investigate the associated fractionation of N isotopes that is expected to occur (Casciotti et al. 2003). Such mechanistic investigations could potentially shed light on the provenance of sponge nitrification, as isotopic composition has been used extensively to trace sources of nutrients in marine organisms (Marguiller 1997, Samarco et al. 1999, Heikoop et al. 2000, Gartner et al., 2002) and to indicate processing of nitrogen (Naqvi et al. 1998, Sigman et al. 2000). Therefore, characterizing the isotopic composition of the nitrate expelled by sponges is a logical first step to investigating the reaction itself and for characterizing its potential impact on the coral reef food web. This chapter presents 1) a survey of nitrification in sponges that expands the number of known species hosting this process from 7 to 17, 2) multiple lines of direct evidence for microbial conversion of ammonium to nitrate, and 3) isotopic analysis of sponge nitrate from both *in situ* and laboratory incubations.

Methods

Study sites

Samples of ambient water and sponge excurrent water (water exiting the osculum) were collected at Conch Reef (locations in Chapter 2) along the Florida Keys island chain. Our sampling took place in July and August 2005 along a ridge at approximately 15 m depth, where the reef topography is primarily spur and groove formations. Because Conch Reef is a protected area, the live sponges used in our incubation experiments were taken from Three Sisters, approximately 4 m deep.

Sampling of sponge excurrent water

SCUBA divers collected samples of sponge excurrent water in acid-washed (10% HCl) 60 ml syringes fitted with a stopcock and a short length of narrow tubing. Fluoroscein dye was used to confirm that the sponge was pumping before the sample was taken. In order to obtain water from the excurrent plume only, excurrent samples were collected slowly (~ 2 ml s^{-1}), and the syringe was flushed with sample first before the final collection. Samples were kept on ice until brought back to the lab, where they were filtered with glass fiber syringe filters (Whatman GF/F, nominal porosity 0.7 μm) and frozen in acid-washed polypropylene centrifuge tubes for nutrient analysis.

We performed qualitative tests of nitrate production on 18 common sponge species. Sponges were considered to host nitrification if excurrent water had significantly higher concentrations of nitrate compared to ambient water adjacent to the sponge individual ($n = \leq 3$) or if nitrate concentrations increased significantly during laboratory incubation experiments with live sponges. Statistical significance for excurrent samples was determined

by using a 2-tailed t-test assuming unequal variance and for incubation experiments by linear regression of nitrate concentration versus time.

Analysis of nitrate isotopic composition required larger samples, so 300 ml samples of sponge excurrent water were collected in 60 ml syringes. Samples were kept on ice until brought back to the lab and filtered as described above. Aliquots of 50 ml were frozen at -20^{o}C in acid-washed polypropylene centrifuge tubes for analysis of nitrate plus nitrite concentration, and the rest frozen at -20^{o}C in acid-washed 250 ml HDPE bottles for analysis of nitrate isotopic composition.

Incubation experiments

SCUBA divers cut pieces of sponge from healthy adults (2-8 cm^{3}, depending on species), attached them to PVC plates with plastic cable ties, and then left the plates attached to platforms out on the reef to recover. Sponges recovered on a time scale of days to weeks, depending on species. Only sponges with healthy appearance (i.e., no visible wounds or decay) were selected for incubation experiments. These sponges were incubated in 2 L HDPE containers of aerated seawater for 6-8 hrs. At no time were the sponges exposed to air. Two aliquots of water were taken at each time point; one was analyzed immediately for ammonium, and the other was frozen in an acid-washed 50 mL polypropylene centrifuge tube for nitrate and nitrite analysis approximately one month later.

Nutrient analysis

Ammonium was measured by fluorescence following the method of Holmes et al. (1999). Briefly, 3 mL of o-phthalaldehyde working reagent was added to 12 mL of sample in an

acid-washed 15 mL centrifuge tube. Samples were incubated in the dark for 2 hours, and then analyzed using a Turner Designs fluorometer, model TD-700, fitted with an ammonium optical kit. Ammonium standards were made fresh each day, and measured along with samples. The limit of detection for ammonium was determined to be 0.2 μmol L^{-1} by repeated measurement of standards. Nitrate and nitrite were measured using standard colorimetric methods on a QuickChem flow-through autoanalyzer (Strickland and Parsons 1972). The limit of detection for nitrate plus nitrite was determined to be 0.25 μmol L^{-1} by repeated measurement of standards, and standard deviation of triplicate samples was 0.17 μmol L^{-1} for both ammonium and nitrate.

^{15}N tracer test for nitrification

Sponges were screened for visible signs of necrosis, and visually healthy individuals were incubated in natural seawater amended with $^{15}NH_4^+$ to a final concentration of 0.2 μM, which was approximately 20% of starting ammonium concentrations (determined in the field). After 8 hours, 50 mL water samples were collected and frozen in acid-washed centrifuge tubes for analysis of the concentration and isotopic composition of NO_3^-.

Inhibition of nitrification

Nitrapyrin (commercial name N-Serve) blocks ammonium oxidation (by bacteria), the first of two steps in the conversion to nitrate (Costa et al. 2006 and references therein). Therefore the difference in ammonium flux with and without N-Serve is taken as a measure of gross nitrification rates. The difference between net nitrate release rates from controls and gross nitrification is assumed to be nitrate uptake. Nitrapyrin is insoluble in water, so it was

first dissolved in dimethyl sulfoxide (DMSO) before it was added to the incubation chambers at a final concentration of 150 μM. *A. cauliformis* and *S. aurea,* two sponges that release nitrate, were incubated in three parallel treatments: DMSO plus N-Serve, DMSO only, and no additions (n=3 for each treatment). Sampling procedures were as described above for incubation experiments.

Nitrate isotopic composition

Samples for determination of the $\delta^{15}N$ value of nitrate were collected at the end of incubation experiments (with and without $^{15}NH_4^+$ added), and also *in situ* from the excurrent plumes of sponges as described above. Samples with < 0.5 μM nitrate were evaporated to dryness on a rotovap, then redissolved in deionized water using the minimum amount of water necessary. Analysis of $\delta^{15}NO_3^-$ values was done using the method of Sigman et al. (2001) in the Stable Isotope Biogeochemistry Laboratories at the University of Hawaii, Manoa. This method uses the denitrifying bacteria *Pseudomonas chlororaphis*, which lacks the enzyme to convert N_2O to N_2. Briefly, 20-mL headspace vials containing pure cultures of *P. chlororaphis* were capped with a Viton stopper and flushed with N_2 or He for 2 hours. An aliquot of the sample was then injected into the vial (2-8 ml, depending on NO_3^- concentration, previously determined) and the culture was allowed to incubate in the dark overnight. The culture was then killed by addition of 0.2 ml of 10 N NaOH. The isotopic composition of the N_2O in the headspace was then analyzed by GC-MS using methods described by Popp et al. (1995) and Dore et al. (1998). Repeated injections were performed on a subset of samples (13%) to evaluate instrumental precision, and analysis of duplicate samples was performed on 19% of samples to evaluate overall sampling and analytical error.

The standard deviation of duplicate injections of samples was 0.15‰, and the standard deviation of duplicate samples was 0.33‰.

Results

Nitrate was produced by 12 of 18 sponge species tested and included the genera *Ircinia, Aplysina, Xestospongia, Pseudoceratine, Smenospongia, and Agelas*. Genera *Callyspongia, Niphates, Spheciospongia,* and *Amphimedon* produced ammonium rather than nitrate (Table 5.1). Presence or absence of nitrification was consistent within a genus. Representative data is shown in Figures 5.1 and 5.2.

N-Serve clearly inhibited nitrification in *A. cauliformis* and *S. aurea*, as ammonium accumulated rather than nitrat (Figure 5.3). DMSO alone had no apparent effect on the production of NO_3^- in *A. cauliformis*, but had a significant effect on NO_3^- production in *S. aurea* (Table 5.2). The partial inhibition in the DMSO control for *S. aurea* may have been caused by cross-contamination with the N-Serve inhibitor, as ammonium-oxidizing organisms can be sensitive to this chemical at extremely low concentration (B. Popp, pers comm.) For *S. aurea*, the rates of total DIN release for the N-Serve, DMSO only, and controls were not statistically different, whereas for *A. cauliformis*, N-Serve and DMSO treatments showed marginally higher rates ($p=0.05$) of DIN release compared to non-treated sponges (Table 5.2).

Nitrate from $^{15}NH_4Cl$ amended incubation experiments was highly enriched in ^{15}N for both *A. cauliformis* and *S. aurea* (Table 5.3). The nitrate recovered from the *A. cauliformis* incubation ranged from 0.46-0.83 atom% ^{15}N, and nitrate from the *S. aurea* incubation

ranged from 0.18-0.32% ^{15}N. Replicate analysis of samples had a standard deviation of 0.04%. With *A. cauliformis*, an average of 14% of the ^{15}N labeled NH_4^+ was recovered as nitrate, whereas an average of 37% was recovered as nitrate in experiments with *S. aurea*.

The $\delta^{15}N$ values of nitrate collected from incubation experiments in which no isotopic tracer was added (i.e. natural abundance) ranged from -6.2 to 5.2‰ (Table 5.4). There was a negative correlation between $\delta^{15}N$ values of nitrate and the fraction of ammonium remaining (Figure 5.4). Nitrate from *in situ* sampling of the sponge excurrent plumes ranged from -0.66 to 3.44‰, with an average of 1.5‰ ±1.4 (Table 5.5). Nitrate in the ambient water column had a $\delta^{15}N$ of 4.2 ±0.8‰ (n=3).

Discussion

Chemical Inhibition

The results of the N-Serve inhibition experiment are consistent with the presence of bacterial nitrification in sponges; however, it is still unknown whether AOA are sensitive to this chemical as well. The total DIN fluxes from DMSO-treated and N-Serve-treated sponges were not statistically different indicating that uptake of metabolically-produced nitrate by internal microbial community is negligible. N-Serve treatments of *A. cauliformis* had marginally higher (p=0.05) DIN flux rates compared to controls. However, the DMSO treatment was similarly elevated therefore this effect could be due to stress caused by the addition of chemicals rather than nitrate uptake in the controls. It is surprising that *A. cauliformis* does not appear to retain internally produced nitrate; this sponge contains chlorophyll a and phycoerythrin in the ectosome, indicating a layer of cyanobacteria in this

sponge (Chapter 3). This result suggests that, if the cyanobacteria in *A. cauliformis* do retain a portion of metabolically produced DIN, then ammonium is the preferred substrate rather than nitrate.

^{15}N tracer

The ^{15}N-labeled ammonium added to the incubations was converted into ^{15}N-labeled nitrate, demonstrating that sponge-associated microbes oxidized ammonium from the ambient water as well as metabolically produced ammonium. The species-specific difference in ^{15}N enrichment of nitrate was probably due to different rates of metabolic production of ammonium by the sponges that diluted the labeled tracer. *S. aurea* is a larger sponge with a high respiration rate (C.S. Martens unpub. data) and therefore probably diluted the tracer to a greater extent. The relatively low recovery of the added ^{15}N as nitrate for *A. cauliformis* (10.6-19.1%) could be due to lower rates of nitrification in this sponge, or to assimilation of ammonium by associated microbes. As discussed above, the layer of photoautotrophs in the outer tissue of *A. cauliformis* are likely candidates for uptake of ammonium.

Natural abundance δ^{15}N

The natural abundance isotopic composition of nitrate produced by sponges during incubation experiments was influenced by the fraction of ammonium oxidized, in accordance with Rayleigh distillation. Although the known mechanism for oxidation of ammonium to nitrate is a two-step process, all isotopic selectivity should occur in the first step if no nitrite intermediate accumulates (Casciotti et al. 2003), therefore the data may be modeled as a single reaction. Both the unreacted ammonium and the nitrate product were retained in the

incubation vessel, so the reaction might be expected to behave as a closed system, in which $\delta_{nitrate} = \delta_{ammonium} - \varepsilon \frac{f(\ln f)}{1-f}$, where f = the fraction of ammonium remaining and ε = the fractionation factor for the reaction (Mariotti et al. 1981). However, the ammonium substrate was produced over time by the remineralization of organic matter, so the reaction could also be argued to also have characteristics of an open system at steady state, in which $\delta_{nitrate} = \delta_{org} + \varepsilon f$ (Hayes 2001). In reality, the reaction probably represents an intermediate situation. Using the closed system model produced a slope (ε) of approximately 11‰ ± 2.6 standard error (S.E.) and a y-intercept of 3.7‰ ± 0.98 (S.E.), with an r^2 of 0.51 (Figure 5.5). The steady state model produced a slope of 14‰ ± 3.5 (S.E.), an intercept of 2.5‰, ± 0.8 (S.E.), and an r^2 value of 0.48 (data not shown). Therefore, whether modeled as a closed or steady state system, approximately half of the variability in the isotopic composition of the nitrate produced by sponges during incubation experiments (with no ^{15}N addition) can be explained by Rayleigh distillation of the ammonium substrate. Because of the variability in the regressions, the fractionation factors and y-intercepts produced by the two models are not statistically different. For the purposes of further discussion, however, I will use the values produced by the closed system model because it more closely resembles the experimental conditions.

The Rayleigh equation applied to a closed system produces an ε value of 11‰ ± 2.6, which is lower than those previously reported for oxidation of ammonium (14 - 38‰, Casciotti et al. 2003). However, given the large 95% confidence intervals associated with this measurements (± 5.1‰), this result is not statistically different from the bacterial values. It is in the lower range of values, which could indicate that the reaction is partially diffusion

limited, or that this microbial community fractionates slightly differently from the bacteria previously studied. Although the fractionation factor for AOA has not been determined, many of the enzymes associated with ammonia oxidation appear analogous to those found in bacteria (Hallam et al. 2006). Therefore the ε value for AOA may be similar to the bacterial range. The y-intercept corresponds to a $\delta^{15}N$ value of 3.7‰ ± 0.98 for the starting pool of ammonium, which is a reasonable isotopic composition for ammonium derived from the catabolism of marine organic matter (Fogel and Cifuentes 1993).

The Rayleigh model explained only 51% of the variability in the dataset. However, these data are from 4 different sponge species, which may host different microbial communities, and have different pumping and respiration rates. Therefore, some of the unexplained variability in the regression may be due to differences in microbial communities, or to differential rates of ammonium assimilation prior to nitrification.

To obtain an environmentally relevant $\delta^{15}N$ value for sponge-produced nitrate, it was necessary to determine the $\delta^{15}N$ value of the nitrate produced *in situ.* While the nitrate in the sponge excurrent plumes ranges from -0.66 to + 3.44 ‰, this could include some contribution of nitrate from the ambient water column, which may be transported passively through the sponge tissue into the excurrent plume. If no ambient nitrate is assimilated as it passes through the sponge tissue, then an isotopic mass balance can be calculated to subtract the contribution of ambient nitrate from the excurrent plume:

$$C_{sponge}\delta^{15}N_{sponge} + C_{ambient}\delta^{15}N_{ambient} = C_{excurrent}\delta^{15}N_{excurrent}$$

The values for the concentration and isotopic composition of the ambient nitrate are based on samples taken on 3 different days during the study (0.8 μM $\pm$ 0.1, 4.2‰ $\pm$ 0.8, n=3). When this calculation is done, the $\delta^{15}N$ of pure sponge-produced nitrate ranges from -

19.4 to 1.8‰, averaging -5.1 $\pm$ 6.4‰. The assumption that ambient nitrate is not assimilated by sponge microbes is supported by the results of the N-Serve inhibition experiment, which demonstrate that no significant uptake of metabolically-produced nitrate is occurring in the two species tested (*A. cauliformis* and *S. aurea)*. Nevertheless, if a portion of the ambient nitrate is taken up, then the actual $\delta^{15}N$ of sponge-produced nitrate must be an intermediate value between the measured $\delta^{15}N$ values of the excurrent plume and the theoretical calculated values for sponge-produced nitrate. Because of the small difference in excurrent nitrate compared to ambient nitrate in some sponges, the uncertainty associated with some of the calculated values is very large (Table 5.5). Despite the large uncertainty, the low adusted values (down to -19‰), are not unreasonable because ammonium oxidation by bacteria strongly selects for the lighter isotope. Previously measured fractionation factors are as great as 38‰ (Casciotti et al. 2003), and data from this study suggests that ammonia oxidation occurs in sponges with a fractionation factor of approximately 11‰. Therefore, catabolism of particulate organic matter followed by partial nitrification could reasonably produce $\delta^{15}N$ values in the range of the adjusted values (-19.4 to 1.8‰). Furthermore, even without the adjustment for ambient nitrate (and its associated uncertainty) the $\delta^{15}N$ values of nitrate in the sponge excurrent plumes are all lower than those of samples from the ambient water. Sponge produced nitrate, therefore, appears to have relatively low $\delta^{15}N$ values.

The variability in $\delta^{15}N$ values of the *in situ* nitrate samples may be controlled by the fraction of ammonium that is consumed by ammonium oxidizers (as was demonstrated in the incubation experiments). The isotopic composition of the nitrate could also be influenced by other microbially-mediated N transformations such as denitrification and DIN uptake. Significant nitrate uptake is not supported by the results of the N-Serve inhibition

experiment; however, uptake of ammonium is likely. Fractionation of ammonium during uptake could thus increase its $\delta^{15}N$ value before nitrification occurs. The nitrate may be further enriched in ^{15}N if denitrification occurs in the low-oxygen zones of sponge tissue (Chapter 5). However, the highest $\delta^{15}N$ value for sponge nitrate measured was still lower than the ammonia substrate predicted by the Rayleigh distillation model from the incubation experiments (1.81‰ in *X. muta* versus 3.7‰ predicted for ammonia). Therefore, in the absence of additional data supporting denitrification or DIN uptake, the range of values measured for nitrate isotopic composition is most reasonably explained by Rayleigh distillation of the ammonium substrate.

Importance of sponge nitrification

DIN flux measurements from 14 sponge species with biomass measurements from a 600 m^2 area of Conch Reef, Key Largo, FL showed that 14 sponge species comprising 85% of the non-encrusting sponge community expel 490 $\pm$ 91 $\mu mol\ m^{-2}\ h^{-1}$ of NO_x^- (Chapter 4). As productivity on coral reefs can be N-limited (Larned 1998), it is important to consider the fate of nitrate released by sponges. Approximately 85% of the sponge nitrate flux is produced by *X. muta*, a massive barrel sponge (Chapter 4). The $\delta^{15}N$ value of nitrate produced by *X. muta* (adjusted for ambient nitrate) was approximately 1‰, and other species produced nitrate that was even more depleted in ^{15}N. Yet, despite this large flux, the ambient nitrate in the water column did not reflect the isotopic composition of sponge nitrate. There are two possible explanations for this phenomenon: 1) the sponge nitrate was diluted by a flux of nitrate with a high $\delta^{15}N$ value from an additional source or 2) sponge nitrate was removed from the water column via processes that selectively use the light isotope, leaving

the remaining pool enriched in ^{15}N. Intrusion of nutrient-rich deep water is known to deliver a significant amount of nitrate to the Florida Keys reef tract (Leichter et al. 2003), and benthic algae exposed to this upwelling have an isotopic composition similar to that of deep water nitrate (5‰, Sigman et al. 2000). By isotopic mass balance, upwelling would have to contribute nitrate at four times the rate of sponges in order to maintain ambient nitrate with a $\delta^{15}N$ value averaging 4.2 ‰. While this is theoretically possible, it is unlikely because the samples were obtained in winter at a depth of ~ 15m, and deep water intrusions rarely reach this depth during the winter months (Leichter and Miller 1999). The more probable explanation is that sponge-produced nitrate is actively utilized by primary producers and denitrifiers, and that these processes enrich the remaining ambient nitrate pool in ^{15}N. By using a steady state model: $\delta_{amb} = \delta_{sponge} + f\varepsilon_{assimilation}$ (Hayes 2001), where δ_{amb} is the $\delta^{15}N$ of nitrate in the ambient water, δ_{sponge} is the $\delta^{15}N$ value of sponge-produced nitrate, f is the fraction assimilated, and ε is the fractionation factor, the fraction of sponge-produced nitrate consumed can be estimated. Assuming a fractionation factor of 5‰ for nitrate assimilation (Fogel and Cifuentes 1993), the model predicts that 64% of sponge-produced nitrate is assimilated. By extension, the $\delta^{15}N$ value of the assimilated nitrate can be calculated according to: $\delta_{biomass} = \delta_{sponge} - (1-f)\varepsilon_{assmilation}$ (Hayes 2001). Using this equation, the $\delta^{15}N$ of assimilated nitrate is approximately -0.8‰. SPOM samples from offshore sites had a mean $\delta^{15}N$ of 2.4 ± 0.03‰. This higher value is not surprising because SPOM represts a mixture of autotrophic, heterotrophic, and detrital particles.

Nitrification appears to be very common in sponges, and our experiments showed that nitrification occurred in all species classified as HMA sponges. This apparent ubiquity of nitrification in HMA sponges and the similarities in microbial community composition

among sponges (Hentschel et al. 2006) supports the hypothesis that this association is symbiotic. The exact nature of the benefits to the respective partners is largely unknown however autotrophic partners such as nitrifying bacteria may provide an additional source of carbon for the sponge. Although the rates of nitrification in sponges are high compared to other benthic environments (Diaz and Ward 1997), the potential carbon fixation rates are insignificant relative to respiration rates and rates of photosynthesis by photoautotrophs (Wilkinson 1983), owing to the inefficiency of nitrification in terms of carbon fixation.

Conclusions

The rates of nitrification measured in this study are consistent with previously reported sponge nitrification rates (Corredor et al. 1988, Diaz and Ward 1997, Chapter 4), which far exceed other nitrification rates on coral reefs (Capone et al. 1992). Webb and Wiebe (1975) showed that coral reef pavement encrusted with cyanobacteria had a gross nitrate production rate ranging from 2.19 to 15.8 μmol $m^{-2}hr^{-1}$ during daylight hours. Based on DIN flux and biomass measurements of 14 common species, sponges occurring on Conch Reef produce 490 $\pm$ 91 μmol NO_3^- $m^{-2}hr^{-1}$ (Chapter 3). This is likely an underestimate because it does not include the 15% of sponge biomass for which DIN flux was not measured, nor does it include encrusting sponges, which can be a large source of nitrate (242 to 454 μmol $m^{-2}hr^{-1}$, Diaz and Ward, 1997).

The apparent fractionation factor and the sensitivity to N-Serve are consistent with bacterially mediated nitrification. However, nitrification by crenarcheota is a relatively recent discovery (Mulder et al. 1995, van de Graaf et al. 1995), and, to my knowledge, little

is currently known about its isotopic fractionation or its sensitivity to chemical inhibition. As nitrifying crenarchaeota have now been successfully cultured (Konneke et al. 2005), this information will likely be available soon, and could provide a means to differentiate bacterial from archaeal nitrification in sponges.

The difference between the N isotopic composition of sponge nitrate and ambient nitrate suggests that this nutrient source is actively utilized and processed after leaving the sponge. Comparison of the $\delta^{15}N$ values of nitrate produced by different sponge species and under different environmental conditions may provide further clues to the mechanisms and controls on sponge-hosted nitrification and the microbial ecology involved in sponge N cycling. The metabolic interactions within the microbial community, as well as oxygen concentration (Muller et al. 2004) potentially complicate the overall net effect of the sponge on rates of N cycling.

Tables

Species	Method	Nitrification	Type
Agelas conifera	Inc/*In situ*	+	HMA
Amphimedon compressa	Incubation	-	LMA
Aplysina archerii	*In situ*	+	HMA
Aplysina cauliforms	Incubation	+	HMA
Aplysina fistularis	Incubation	+	HMA
Aplysina lacunosa	*In situ*	+	HMA
Callyspongia plicifera	*In situ*	-	LMA
Callyspongia vaginalis	*In situ*	-	LMA
Chondrilla nucula	Incubation	+	HMA
Ircinia campana	Incubation	+	HMA
Ircinia felix	Incubation	+	HMA
Ircinia strobilina	*In situ*	+	HMA
Niphates digitalis	*In situ*	-	LMA
Niphates erecta	Incubation	-	LMA
Pseudoceratina crassa	*In situ*	+	HMA
Smenospongia aurea	Inc/*In situ*	+	HMA
Spheciospongia vesparium	*In situ*	-	HMA
Xestospongia muta	Inc/*In situ*	+	HMA

Table 5.1. Qualitative results from nitrification survey.

Species	**Treatment**	**NO_3^-** $\mu mol\ g^{-1}\ hr^{-1}$	**NH_4^+** $\mu mol\ g^{-1}\ hr^{-1}$	**$NO_3^- + NH_4^+$** $\mu mol\ g^{-1}\ hr^{-1}$
A. cauliformis	Control	1.65 (0.51)	-0.54 (0.46)	1.11 (0.06)
A. cauliformis	DMSO	1.5 (0.49)	0.35 (1.01)	1.85 (0.52)
A. cauliformis	N-Serve + DMSO	-0.07 (0.06)	2.16 (0.37)	2.09 (0.42)
S. aurea	Control	1.84 (0.26)	0.04 (0.02)	1.87 (0.28)
S. aurea	DMSO	0.79 (0.25)	0.29 (0.14)	1.38 (0.29)
S. aurea	N-Serve + DMSO	0.02 (0.01)	1.49 (0.14)	1.51 (0.13)

Table 5.2. Average flux rates from N-Serve inhibition experiment. Numbers in parentheses are standard deviation.

Species	$\delta^{15}N$	Atom % ^{15}N	% tracer recovered as NO_3^-
A. cauliformis	2271	0.83	19
A. cauliformis	1255	0.46	10
A. cauliformis	1313	0.48	12
S. aurea	778	0.29	43
S. aurea	879	0.32	37
S. aurea	482	0.18	28

Table 5.3. Isotopic composition of nitrate after 8 hr. incubation of sponge with 0.2 μM $^{15}NH_4^+$ tracer.

	$\delta^{15}N$	$[NH_4^+]$	$[NO_3^-]$	$[NH_4^+]/([NO_3^-]+[NH_4^+])$
Ircinia felix	-6.20	14.82	64.62	0.19
Ircinia felix	5.17	1.55	31.47	0.05
Ircinia felix	-1.03	5.81	29.40	0.17
Smenospongia aurea	3.17	2.68	93.00	0.03
Smenospongia aurea	-4.85	13.36	20.31	0.40
Smenospongia aurea	-2.79	29.36	35.82	0.45
Xestospongia muta	-0.90	0.70	47.46	0.01
Xestospongia muta	1.91	0.79	15.80	0.05
Xestospongia muta	2.89	0.45	16.20	0.03
Aplysina cauliformis	0.93	4.80	12.88	0.27
Aplysina cauliformis	0.44	2.11	12.85	0.14
Aplysina cauliformis	-0.24	0.40	3.88	0.09
Aplysina cauliformis	3.29	0.85	14.29	0.06
Aplysina cauliformis	2.93	0.74	8.88	0.08
Aplysina cauliformis	0.21	2.65	12.72	0.17
Aplysina cauliformis	1.95	0.98	11.09	0.08
Aplysina cauliformis	-3.06	6.71	5.89	0.53
Aplysina cauliformis	-2.20	3.23	9.29	0.26
Aplysina cauliformis	1.96	0.63	4.52	0.12
Aplysina cauliformis	3.32	3.19	3.08	0.51

Table 5.4. Natural abundance $\delta^{15}N$ values of nitrate collected from incubation experiments after 20 hours.

Species	Net $[NO_3^-]$ μM	$\delta^{15}N$ Excurrent Plume, ‰	$\delta^{15}N$ Sponge NO_3^-, ‰	Propagated SD, ‰
A. archerii	0.49	1.0	-4.5	3.5
A. lacunosa	0.31	0.7	-8.7	8.8
A. lacunosa	0.97	-0.3	-4.3	1.8
I. strobilina	0.30	0.8	-8.6	8.9
I. strobilina	0.43	3.0	0.8	3.1
I. strobilina	0.52	1.7	-2.3	2.6
S. aurea	0.34	-0.7	-12.6	11.0
S. aurea	0.18	0.1	-19.4	31.2
X. muta	0.91	2.5	0.9	1.5
X. muta	0.97	2.9	1.8	1.5
X. muta	0.96	2.2	0.4	1.4
Avg. Ambient (n=3)	0.84	4.2		

Table 5.5. Isotopic composition of nitrate from sponges sampled *in situ.* SD = standard deviation. For propagation of error, standard deviations of nitrate concentrations and $\delta^{15}N$ values were 0.2 μmol L^{-1} and 0.3‰, respectively.

Figures

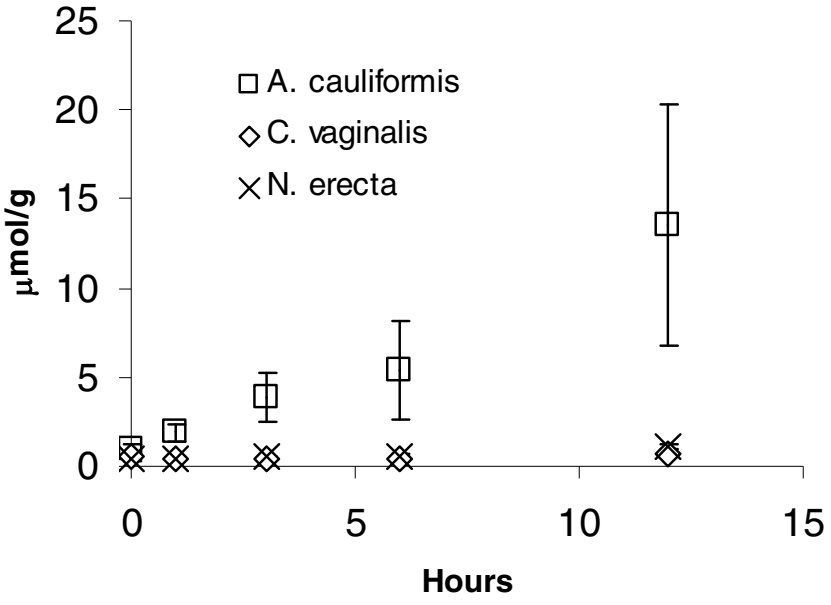

Figure 5.1. Nitrate concentration during incubation experiments. n = 3 for each species, error bars are standard deviation.

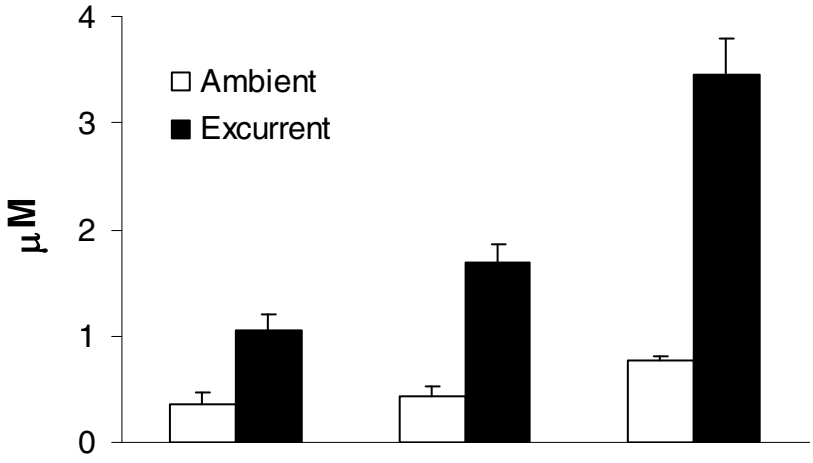

Figure 5.2. ΔDIN for in three individuals of *I. strobilina*. Each set of bars represents an individual sponge, n = 3, $p < 0.01$. Error bars are standard deviation.

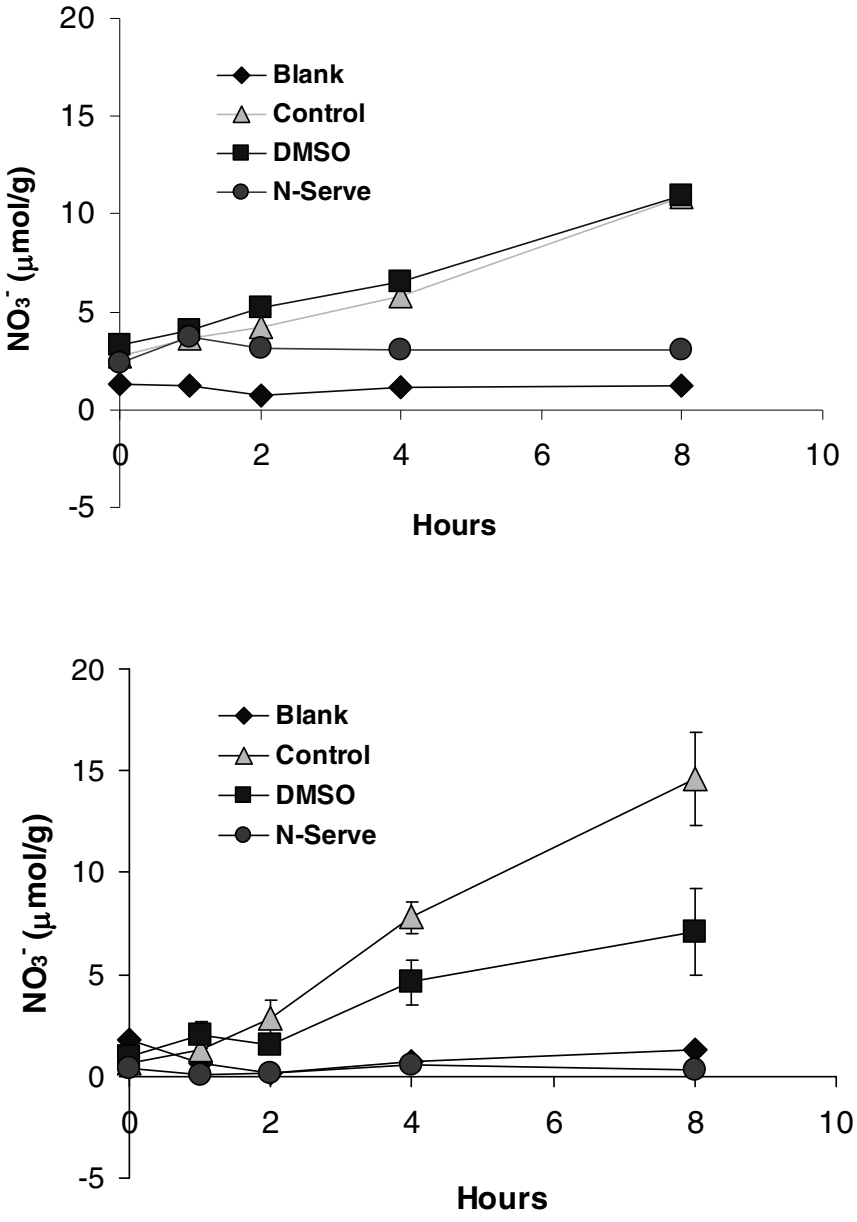

Figure 5.3. N-Serve inhibition with *A. cauliformis* (upper panel) and *S. aurea* (lower panel). Error bars are standard deviation (n=3).

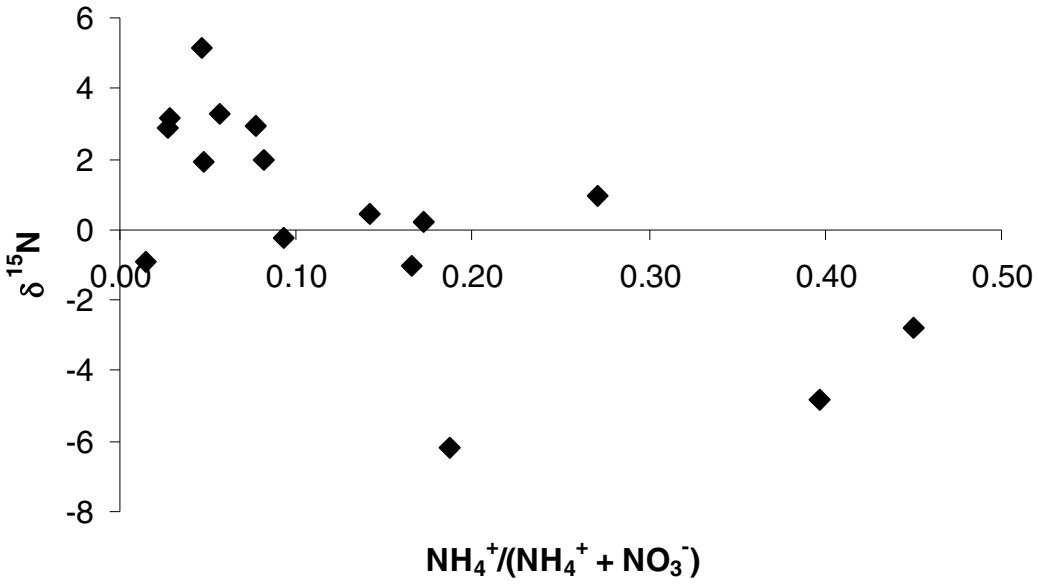

Figure 5.4. Natural abundance $\delta^{15}N$ values of nitrate produced versus fraction of ammonium remaining after 20 hours incubation.

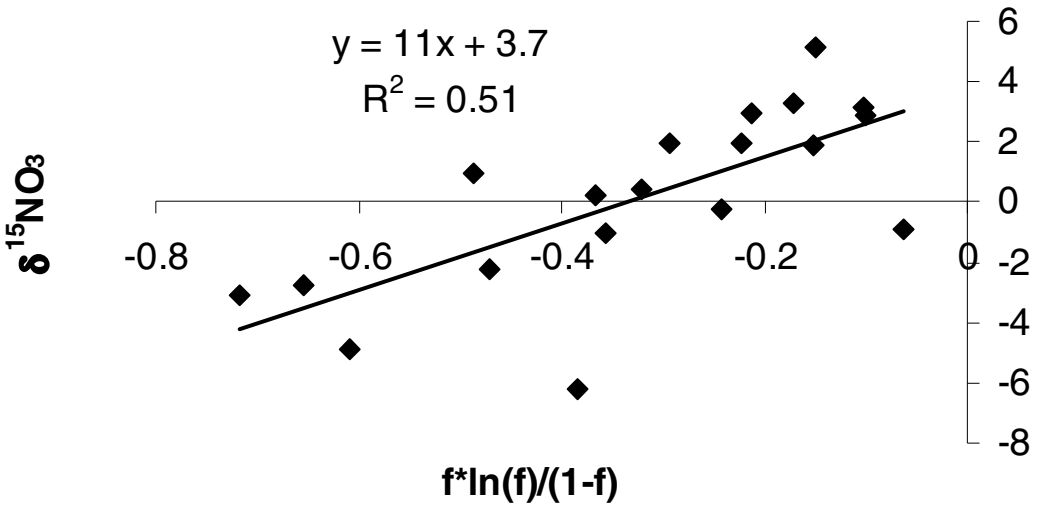

Figure 5.5. Rayleigh distillation model (closed system).

CHAPTER 6

Hypoxia and nitrogen fixation in sponges

A recent survey of C and N stable isotopic compositions of sponge tissue from the Florida Keys showed that a subset of HMA sponges have $\delta^{15}N$ values approximately 3‰ lower compared to other species at the same sites (Chapter 3). Low $\delta^{15}N$ values have often been interpreted as evidence for N_2 fixation (Yamamuro et al. 1995, Samarco et al. 1999) because the isotopic fractionation factor associated N_2 fixation is small (Fogel and Cifuentes 1993) so that newly fixed nitrogen usually has a $\delta^{15}N$ value similar to atmospheric N_2 (~ 0 ‰). Thus, the low $\delta^{15}N$ values of some species (-0.05 to 2.29‰) suggest that they could be obtaining a significant portion of their dietary N from N_2 fixation by the internal microbial community. Given the dense populations of sponges on Caribbean reefs (Wulff 2006, Chapter 4), this phenomenon could constitute a potentially significant source of new nitrogen for an ecosystem that is sensitive to nutrient fluctuations (Lapointe 1997, Larned 1998).

The concept of N_2 fixation by sponge microbes is not new, yet quantitative proof of this process is scarce (Wilkinson and Fay 1979, Shieh and Lin 1994, Wilkinson 1999). Wilkinson and Fay (1979) first measured N_2 fixation using the acetylene reduction technique, however subsequent attempts to use this method have proven problematic (Wilkinson 1999). This could be due to the difficulty in maintaining sponge health in aquaria, or to complications with using the acetylene reduction method on sponges. Acetylene blocks

nitrification and denitrification, and nitrification occurs widely in sponges. Therefore, the acetylene reduction method may not work in sponges because it disrupts other, concurrent N processes.

Many species of HMA sponge have hypoxic or anoxic zones (Hoffman et al. 2005) that could facilitate oxygen-sensitive processes including N_2 fixation. This chapter presents oxygen profiles of several sponge species coupled with data from $^{15}N_2$ assays designed to directly detect N_2 fixation. Tracer experiments were also performed on seawater alone in order to compare the importance of sponge N_2 fixation compared to planktonic N_2 fixation.

Methods

Sample collection

Sponges were obtained from Three Sisters Reef, (locations in Chapter 2). SCUBA divers cut four pieces of sponge from healthy adults (2-8 cm3, depending on species), attached them to PVC plates with plastic cable ties, and then left them on the reef to recover. Sponges recovered on a time scale of days to weeks, depending on species. Two healed pieces from each individual were selected based on healthy appearance (no visible wounds or decay) to represent the initial and final time point of the assay. Seawater for the incubations was also obtained from Three Sisters Reef.

Oxygen profiles

Oxygen profiles in one LMA sponge (*Niphates digitalis*) and three HMA sponges (*Aplysina cauliformis, Agelas conifera,* and *Ircinia felix)* were measured *in situ* using a

Unisense Clark-style needle electrode with a submersible meter. The electrode was mounted on a micromanipulator clamped to a ring stand next to the sponge, and the orientation of the profiles was orthogonal to the sponge surface. The spatial resolution of the electrode is estimated at 1.1 mm (per Unisense) therefore measurements were made at 1mm intervals. Seawater equilibrated with air at *in situ* temperature and anoxic mud was used to provide a two-point calibration of the electrode. The ambient water next to the sponge was also measured, and the oxygen concentrations in the sponge tissue were converted to the percentage difference between internal concentrations and the oxygen concentration in the ambient water adjacent to the sponge.

N_2 fixation measurement by $^{15}N_2$ Assays

Laboratory experiments

Sponges were brought to the field laboratory at NURC, where one piece from each sponge individual was immediately frozen for t = 0. The other pieces were incubated in 4 L gas-tight acrylic vessels for 24 hours with a 60 mL headspace composed of 80% $^{15}N_2$, 20% O_2. This achieved a total enrichment of approximately 50% in the N_2 pool. Controls contained 60 ml of air rather than the tracer-enriched headspace. The seawater in the incubation vessel was either unfiltered, filtered, (cellulose acetate filters 0.2 μm) or artificial seawater made from distilled water and Instant Ocean© salts. The incubations were performed in a shaded area outdoors, and the chambers were submerged in a flowing seawater bath to maintain ambient temperature. Each chamber was fitted with a small power head pump to circulate the water and speed exchange between headspace and dissolved gases. The chambers also had two ports fitted with 3-way valves. Oxygen was monitored

every 4 hours by taking a 10 mL aliquot of water with a syringe while adding an equivalent amount of degassed seawater. A Clark-style needle electrode was then inserted into the syringe tip to measure O_2 concentration. If the O_2 concentration fell below 80% of saturation, the chamber was amended with additional O_2 gas. After 24 hours, the sponge was removed and frozen. It was later freeze-dried, weighed, and prepared for isotopic analysis using methods described below.

Fixation of labeled $^{15}N_2$ into particulate matter would allow sponges to incorporate the isotopic label by filtration rather than fixation by the internal microbial community, so parallel blank experiments with seawater alone were also performed in order to determine potential for N_2 fixation from bacterioplankton in the water column. These experiments were set up as described above, with seawater before and after a 24 hour incubation with $^{15}N_2$ filtered on a pre-combusted GF/F glass fiber filter. These seawater blanks were performed on unfiltered as well as pre-filtered and artificial seawater. In addition to the 2-time point experiments described above, a time course assay was done with unfiltered natural seawater.

Aquarius experiments

Experiments with artificial and natural seawater were also performed *in situ* from the Aquarius underwater laboratory. Artificial seawater was made with Instant Ocean salts and commercially obtained distilled water. Healed sponges were brought into the "wet porch" area of the Aquarius and transferred to chambers filled with artificial seawater. Some ambient seawater had to be transferred with the sponge however this was minimized as much as possible, while keeping the sponge submerged at all times. Control experiments with seawater only (described above) were also performed. The chambers were 3.2 L, and were fitted with a rubber septum, which allowed O_2 concentration to be monitored using a

reinforced needle oxygen electrode and submersible meter (Unisense). A headspace of $^{15}N_2/O_2$ was added as described above. The chambers were then deployed onto a rotating rack in order to circulate the water and expose all sides of the sponge equally to light and to stir the water in the chambers. Oxygen was measured every 4 hours. After 24 hours, the sponges were recovered and frozen.

Isotopic analysis

The sponges and filters were dried in a lyophilyzer, and the sponge samples were then ground with a ceramic mortar and pestle until homogenous. Samples were weighed and vapor-acidified using concentrated HCl (Hedges and Stern 1984) in order to remove any carbonate sand that may have become incorporated into the sponge tissue. C and N stable isotopic analysis of the filters and sponges was performed on a Carlo-Erba Elemental Analyzer coupled to a Finnigan MAT 252 IRMS, in comparison with acetanilide reference material of known isotopic composition.

Statistical treatment

N_2 fixation

The $\delta^{15}N$ values of the initial and final time points of the incubation (t=0 and t=24) were compared for significant differences using a 2-tailed t-test for difference of means with unequal variance ($p<0.05$). The difference between the initial and final $\delta^{15}N$ values for the different species and treatments were then compared also using a 2-tailed t-test.

Results

O_2 profiles

The depths of the profiles were sometimes limited by the thickness of the sponge. *A. cauliformis*, for example, is a thin rope sponge less than 1 cm in diameter, and so the profile is very short. Profiles of all species except for *N. digitalis* showed that concentrations dropped significantly within 2 mm of the sponge surface (Figure 6.1). In all individuals tested except for *N. digitalis*, O_2 was reduced to less than 10% of ambient water within 4 mm of the sponge surface. In *N. digitalis*, oxygen was reduced by approximately 50%. In all *A. archerii*, and one individual of *A. conifera*, O_2 was undetectable within 2 mm of the sponge surface.

N_2 fixation assays

Laboratory experiments

Both *A. cauliformis* and *N. erecta* incorporated a significant amount of ^{15}N tracer during the incubation, irrespective of incubation medium (natural, filtered, or artificial seawater) (Figures 6.2 and 6.3). In all cases, *A. cauliformis* incorporated more ^{15}N than *N. erecta*. However, the amount of ^{15}N fixed onto particulate matter in the seawater controls (4 $\mu g\ L^{-1}\ d^{-1}$) was greater than the amount acquired by either species (Figure 6.4). Time-course measurements of $^{15}N_2$ uptake support rapid N_2 fixation by planktonic bacteria (Figure 6.5). Filtering the seawater reduced the amount of fixation, and using artificial seawater reduced it further (Figure 6.6). Nevertheless, the amount of ^{15}N fixed in 4L of seawater (the volume of the incubation chambers) was always greater than the amount found in the sponge. The

amounts of tracer incorporated by *N. erecta* in natural, filtered, and artificial seawater were not statistically different (Figures 6.2 and 6.3). *A. cauliformis* incorporated significantly less tracer when incubated in filtered seawater compared to natural or artificial seawater. However, the experiment in filtered seawater was conducted in winter, and therefore may have been affected by the lower temperature.

Aquarius experiments

In natural seawater, both *I. campana* and *A. cauliformis* were significantly enriched in ^{15}N after 24 hours of exposure to the $^{15}N_2$ tracer, whereas *N. erecta* was not significantly enriched (Figure 6.7). Given the mass and N content of the sponges, the change in $\delta^{15}N$ translates to 1.4 mg new N g^{-1} for *A. cauliformis*, and 1.4 μg new N g^{-1} for *I. campana*. In artificial seawater, only *A. cauliformis* showed significant enrichment in ^{15}N (Figure 6.7); it incorporated 0.3 μg new N g^{-1}. The results from artificial seawater are compromised, however, because bringing the chambers into the pressurized "wet porch" caused the artificial seawater to be supersaturated with oxygen. Furthermore, the commercially-obtained distilled water used to make the artificial seawater was found to contain approximately 4 μmol L^{-1} of ammonium.

Discussion

O_2 profiles

As expected, O_2 concentrations decreased within the tissue of all the sponge species (Figure 6.1). In *I. felix, A. archerii, A. cauliformis,* and *A. conifera,* O_2 decreased to

undetectable levels in at least one individual; however, because of the difficulty in precisely calibrating the O_2 meter *in situ*, it is not possible to determine whether these sponges were truly anoxic or extremely hypoxic. *N. digitalis,* the only LMA sponge tested had more oxygenated tissues than the other (HMA) sponges tested. *I. felix* has a tough ectosome, and some flexing of the sponge surface was observed before the needle penetrated the outer layer of tissue. Therefore, the depth of O_2 penetration in this sponge cannot be considered precise. However, the profiles generally show that very hypoxic conditions are common in HMA sponges and that oxygen-sensitive reactions such as N_2 fixation, denitrification, and perhaps anammox could conceivable occur in these tissues.

N_2 fixation

Overall, the rates of N_2 fixation in sponges indicated by these results are quite low. It is possible that buildup of DIN in the incubation chambers suppressed N_2 fixation. DIN concentrations were not directly tested in this experiment; however, extrapolating from measured flux rates (Chapter 4), DIN concentrations were probably in the range of 10-30 μL after 24 hours. It is unclear whether this would inhibit N_2 fixation occurring in the sponge interior, where DIN concentrations are probably higher than ambient concentrations under natural conditions. While pure cultures of diazatrophs have been shown to be inhibited by DIN at lower concentrations than these, whole communities *in situ* appear to be much more resistant to inhibition. Furthermore, nutrient buildup in the chamber would take some time, and so N_2 fixers would have been unaffected by this for at least the early part of the incubation.

The use of artificial seawater in saturation proved to be very problematic. The necessity of filling the chambers in the wet porch allowed the artificial seawater to become supersaturated with respect to O_2 (pressure in the Aquarius is ~ 2.5 that of atmospheric pressure). Therefore the low incorporation during saturation experiments with ASW (Figure 6.7) is likely due to inhibition by O_2. The one experiment done in Aquarius with natural seawater had results similar to land-based experiments (Figure 6.7), and was presumably not inhibited by elevated O_2 levels because those chambers were filled outside of the Aquarius and not in the wet porch. The one useful result that can be gleaned from the *in situ* experiments is that the inhibition of N_2 fixation in the hyper-oxygenated experiments demonstrates that the tracer incorporation in the other experiments is likely due to biological N_2 fixation, and not to adsorption of $^{15}N_2$ tracer.

Because the planktonic community was actively N_2 fixation, it is not possible to prove that the ^{15}N obtained by the sponge was fixed internally and not by planktonic bacteria that was subsequently filtered. Sponges are able to filter large volumes of water, and a typical pumping rate for an HMA sponge is approximately 5 times its volume every minute (Weisz 2006). Therefore, the 2.5 μg g^{-1} (or 380 μg per liter of sponge) of ^{15}N tracer acquired by *A. cauliformis* in natural seawater over 24 hours (Figure 6.4) could theoretically be obtained in about 20 minutes by filtering seawater alone. However, if the enrichment in sponge tissue were mostly from filtration of planktonic N_2 fixers, the amount of tracer acquired by the sponge should have been lower for filtered and artificial seawater, echoing the activity of the seawater controls (Figure 6.4).

The consistency of the sponge incorporation, therefore, suggests that this fixation is probably internal and not external. *A. cauliformis* acquired about 1000 times the amount of

^{15}N tracer incorporated into an equivalent volume of seawater particulate matter. In that sense, this sponge did represent a site of accelerated N_2 fixation. However, these rates are consistent with the greater density of microbes in sponge tissue (about 2-4 orders of magnitude higher than seawater, Hentschel et al. 2003). Therefore, N_2 fixation is not apparently accelerated by the hypoxic environment in the sponge interior. Furthermore, it is possible that, though internal, the sponge N_2 fixers may be transient, i.e. recently filtered planktonic bacteria, rather than part of the sponge-specific microbial community. This is supported by the smaller, but consistent incorporation by the LMA sponge *N. erecta*.

Whether the fixation was by sponge-specific or planktonic bacteria, *A. cauliformis* always acquired more "new N" than *N. erecta* (Figure 6.3). This proportionally greater input of newly fixed N could, hypothetically, contribute to the low natural abundance $\delta^{15}N$ values of this sponge (1.9‰ $\pm$ 0.8). Feeding studies show that unicellular bacteria capable of N_2 fixation (e.g. *Synechococcus*) are a significant dietary component of sponges, but that most sponges show little or no selectivity in the types of picoplankton they filter (Pile 1996, 1999). Therefore, the consumption of N_2-fixing bacteria from the water column is not a likely explanation for the differences in natural abundance $\delta^{15}N$ values of different sponge species.

The sponge community on Conch Reef, near Key Largo, FL was found to release an estimated 660 $\pm$ 130 μmol m^{-2} h^{-1} of DIN (Chapter 4). It has been hypothesized that part of this flux is fueled by N_2 fixation (Diaz and Ward 1997); however, my results suggest that internally-hosted N_2 fixation could only fuel a small fraction of this flux. *A. cauliformis* acquired 2.5 μg g^{-1} d^{-1} of N_2 tracer, but releases ~ 430 μg g^{-1} d^{-1} of DIN. It is possible that newly fixed N is not retained by the internal microbial community or the sponge, and that the ^{15}N tracer was re-released as DIN (the $\delta^{15}N$ values of DIN in these experiments was not

determined). However, performing an energetically costly process such as N_2 fixation without the benefit of retaining the product does not make sense. The more likely interpretation of the disparity between N_2 fixation rates and DIN flux rates is that organic matter filtration is of much greater source of N than internal N_2 fixation. If sponges and their microbial communities are able to obtain adequate N from organic matter sources, they are unlikely to resort to the energetically costly process of N_2 fixation. The low rates in this study may, therefore, not be characteristic of sponges in other, more nitrogen-limited regions.

The blank experiments with natural seawater (Figure 6.5 and 6.6) indicated that water column microorganisms are fixing N_2 at a rate of approximately 4 $\mu g\ L^{-1}\ d^{-1}$. For Conch Reef, which is approximately 15 m deep, this translates into 4.5 mmol (as N) $m^{-2}\ d^{-1}$ of new N. There is a wide range of N_2 fixation rates reported from oligotrophic waters, however the rates suggested by the blank experiments in this study are, to my knowledge, the highest areal rates yet reported for water column N_2 fixation in tropical waters (Montoya et al. 2004 and references therein). The 4.5 mmol of new N $m^{-2}\ d^{-1}$ from planktonic diazotrophs could therefore support a significant portion of the 16 mmol DIN $m^{-2}\ d^{-1}$ released by sponges. If sponges reduce the population of diazotrophs in the water column by grazing, then they may reduce water column N_2 fixation rates, and therefore inputs of new N to the reef. Also, the release DIN from sponges could increase nutrient concentrations in the water column, which could further reduce N_2 fixation rates.

Conclusions

The sponges in this study did not exhibit high rates of N_2 fixation. Therefore, the hypothesis that sponges constitute an important source of new nitrogen for the reef is not

supported by these data. This result is surprising, given favorable hypoxic environment in the sponge and the potential ecological advantages of a partnership with an N_2 fixing community. However, such ecological advantages only exist in environments that are nitrogen poor, and it is not certain whether these sponges qualify as such. Therefore, it remains possible that high rates of N_2 fixation in sponges could occur under greater N limitation, and field work in the Great Barrier Reef is suggested to test this hypothesis.

Figures

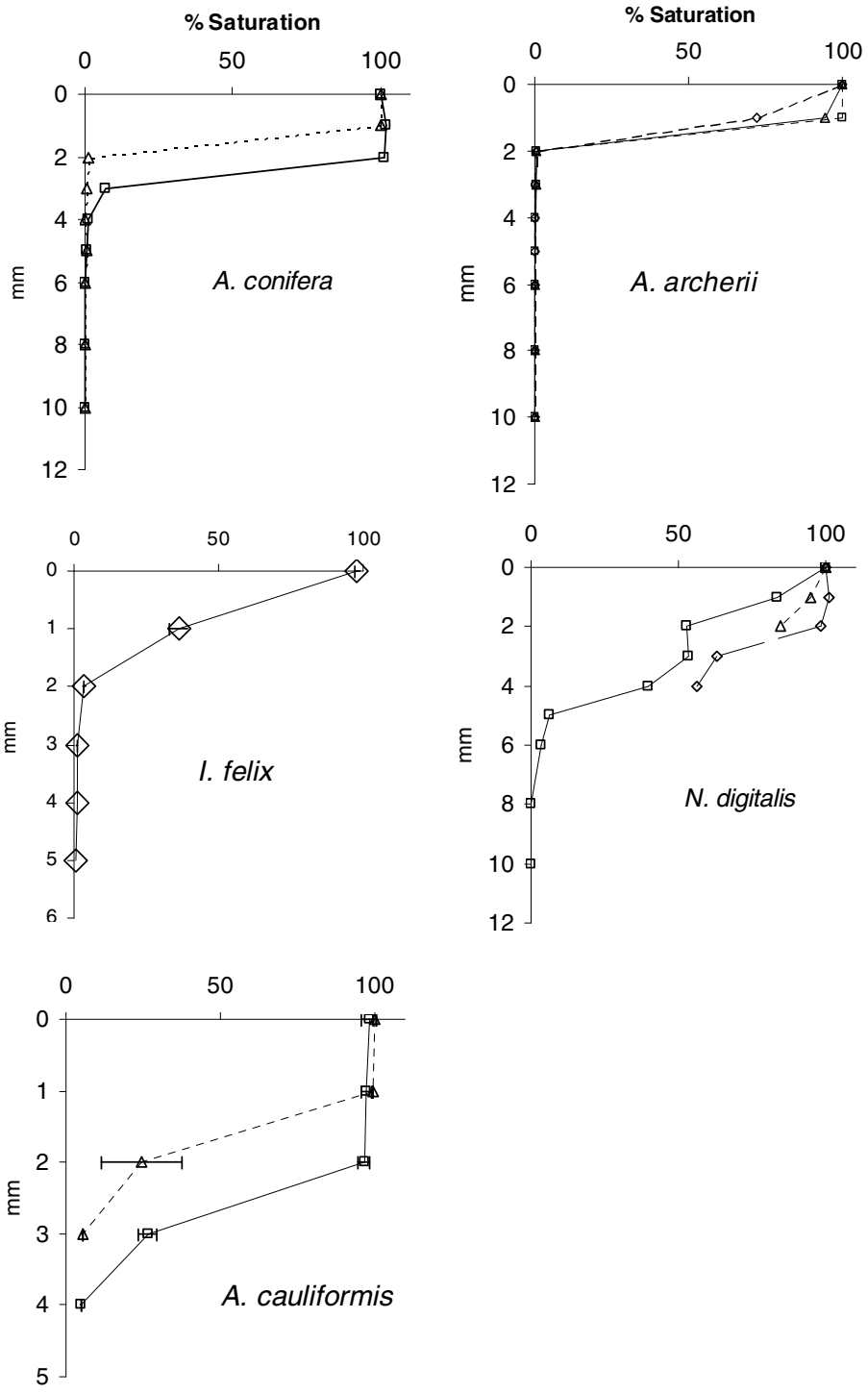

Figure 6.1. Oxygen profiles in sponges. Different symbols represent different individuals, and error bars are standard deviation.

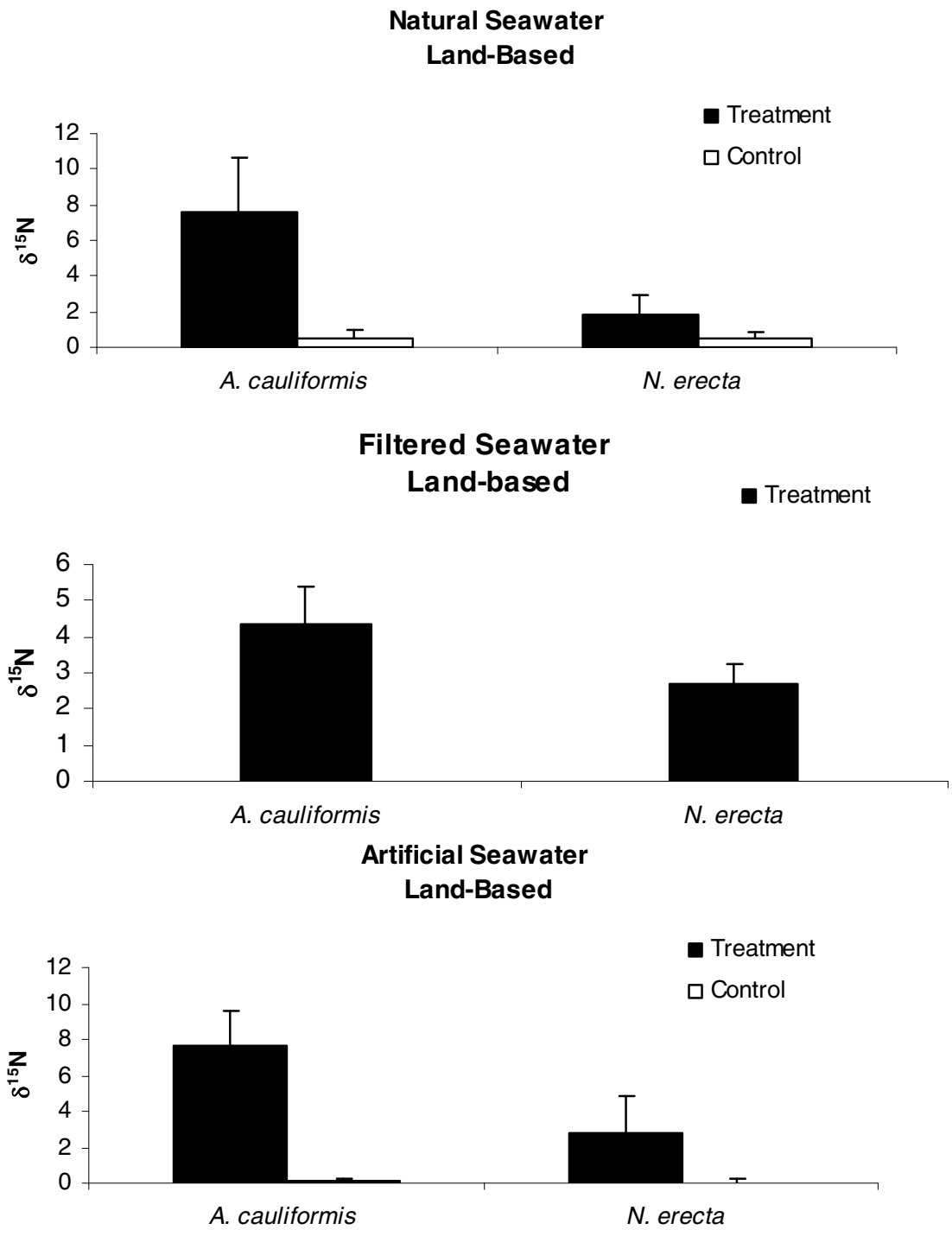

Figure 6.2. N_2 fixation experiments (laboratory based). The difference between t = 0 and t = 24 $\delta^{15}N$ is plotted on the Y axis. Units are ‰, error bars are standard deviation.

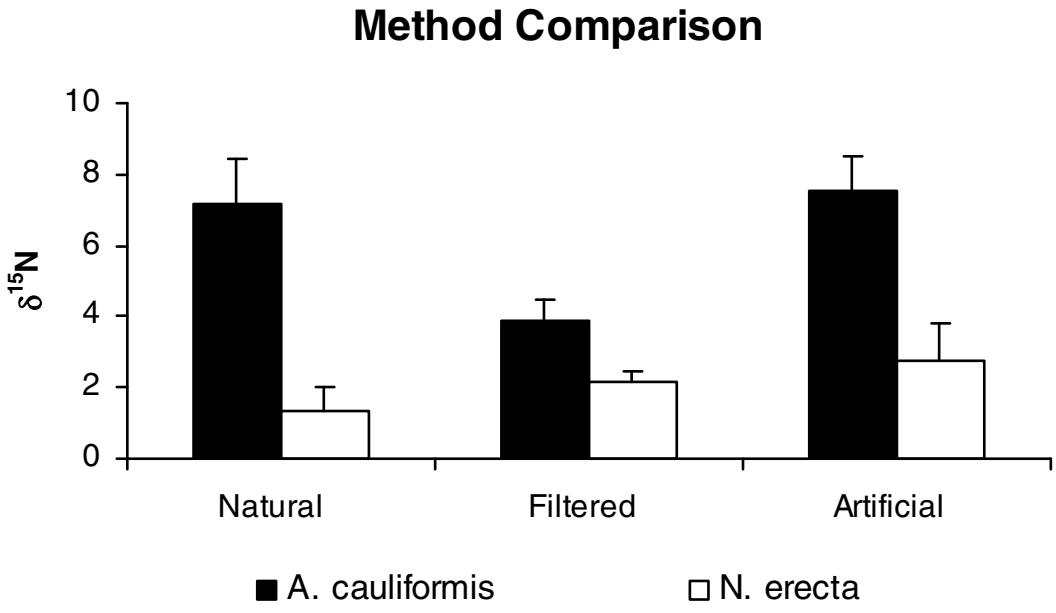

Figure 6.3. Comparison of unfiltered, filtered, (0.2 μm) and artificial seawater (laboratory based only). Difference between $\delta^{15}N$ of t=0 and t=24 is plotted on the Y axis. Units are ‰, error bars are standard deviation.

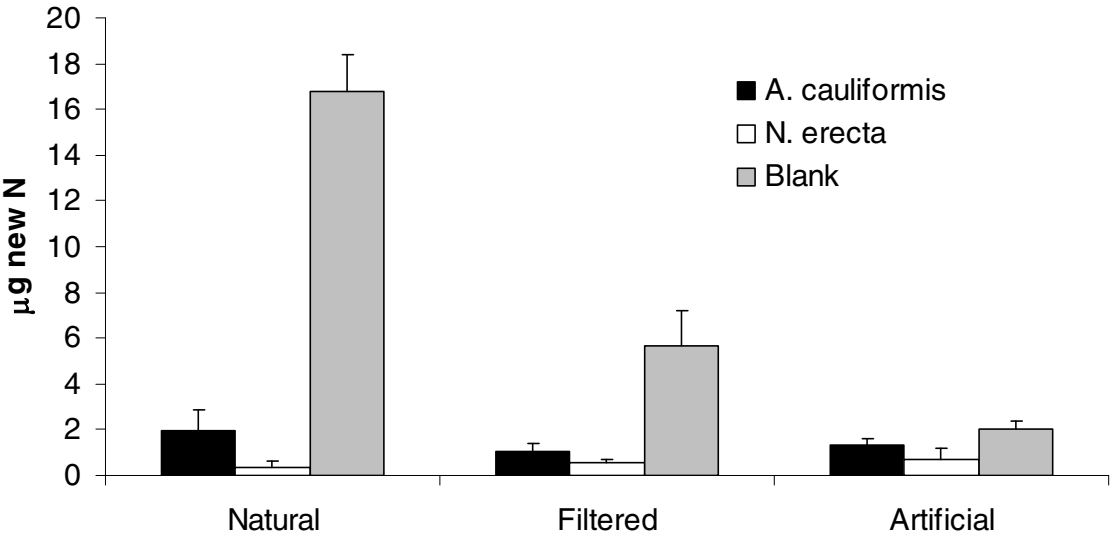

Figure 6.4. Amount of $^{15}N_2$ incorporated into sponge tissue and particulate matter for laboratory based experiments. Blanks are weighted by volume of seawater in the incubation vessel and the sponges are weighted by the dry masses of sponges used in the incubations. Error bars are standard deviation.

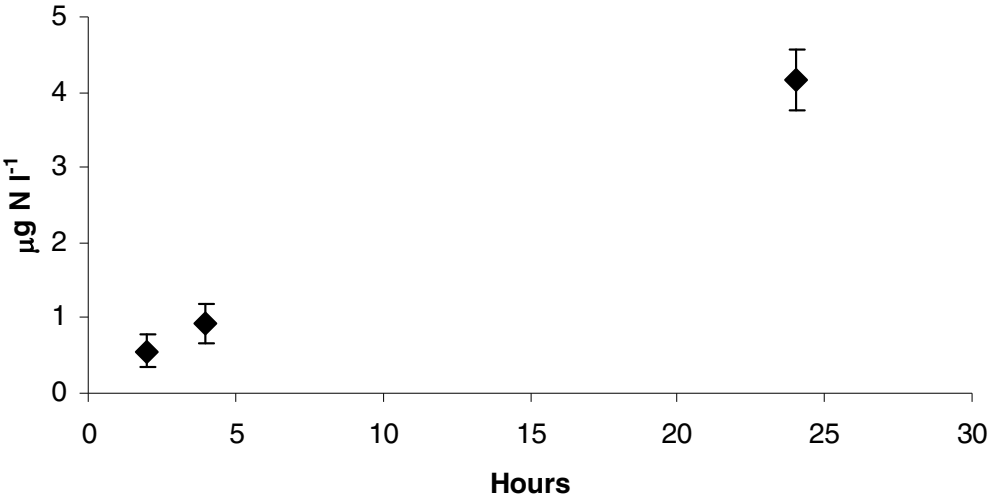

Figure 6.5. Time course of $^{15}N_2$ tracer fixed in blank experiment with natural seawater. Error bars are standard deviation.

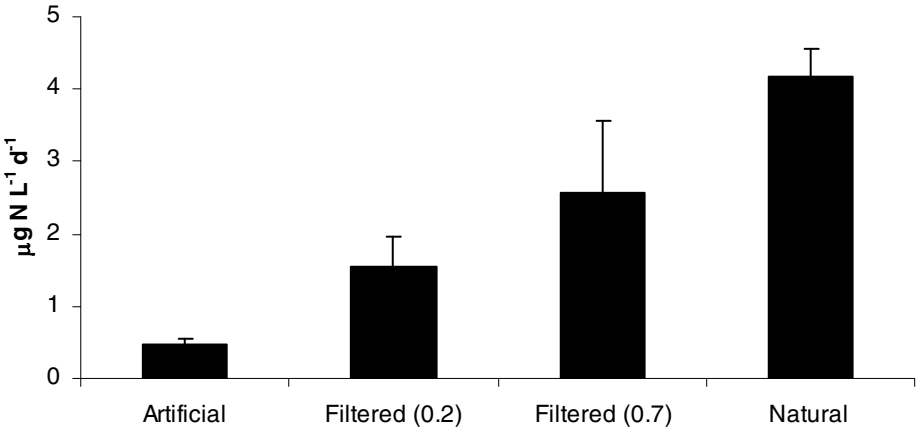

Figure 6.6. $^{15}N_2$ tracer incorporated into particulate matter in blank experiments. Error bars are standard deviation.

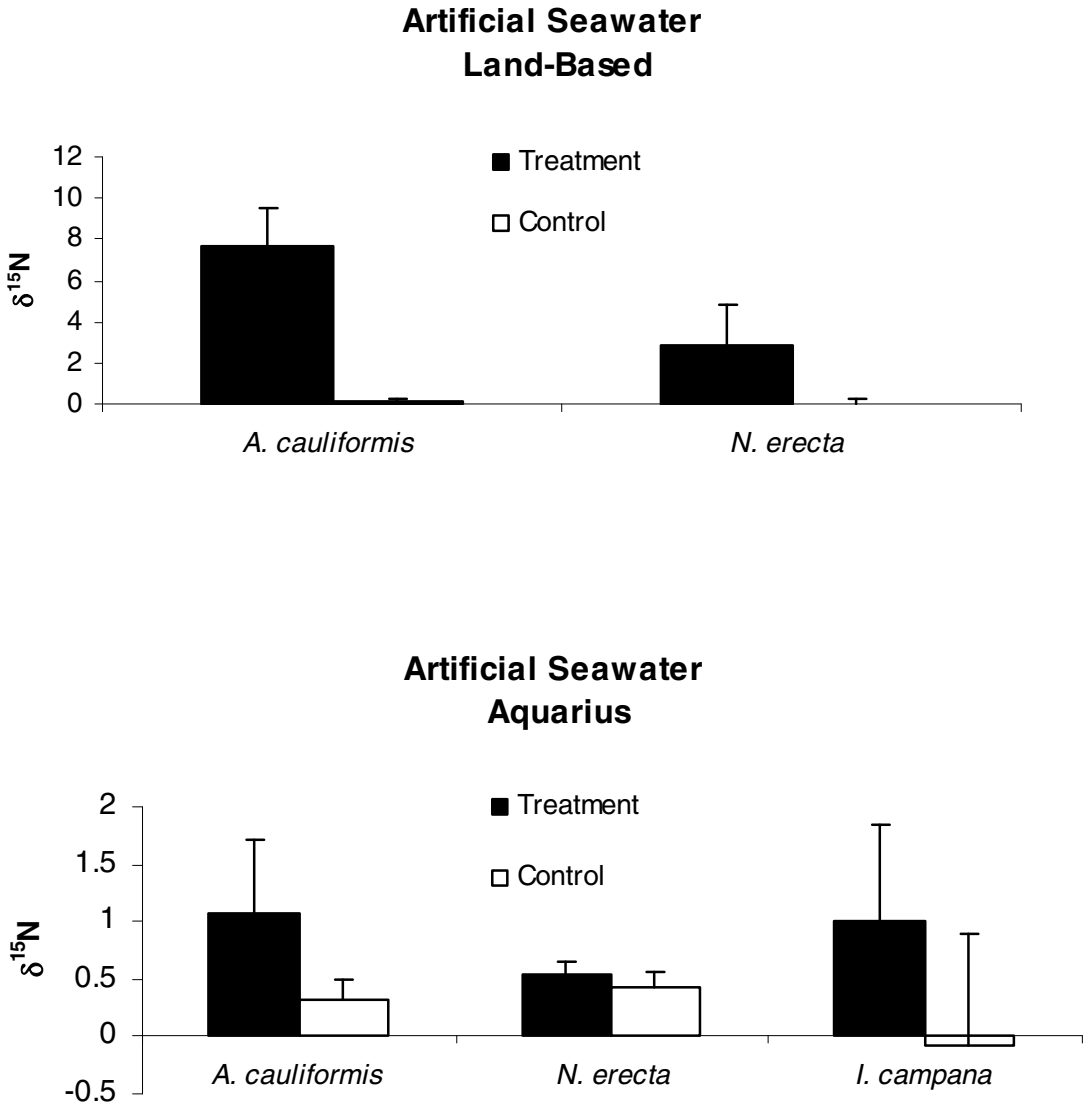

Figure 6.7. $^{15}N_2$ tracer experiments performed at Aquarius. The difference between t = 0 and t = 24 is plotted on the Y axis. Units are ‰, error bars are standard deviation.

CHAPTER 7

Synthesis, speculation, and future directions

Synthesis

The results of this research have provided quantitative information about DIN fluxes from sponges, new insights into sponge nitrification, and new perspectives on the potential (and limitations) for sponges to record highly labile organic matter sources. I have analyzed many different sponge species for isotopic composition, pigment content, and various biogeochemical processes (Table 7.1). The results of these experiments have allowed me to affirm or reject some of the hypothesized processes hosted by sponges (Figure 7.1).

The release of ammonium and nitrate from sponges had been previously reported (Corredor et al., 1988, Diaz and Ward 1997), and the DIN flux was therefore expected; however, the magnitude of the flux was surprising. The measured rate from 14 species, 550 $\pm$ 120 μmol m^{-2} h^{-1}, represents a significant flux of DIN relative to other known benthic sources. Furthermore, the predicted community flux of 660 $\pm$ 130 μmol m^{-2} h^{-1} is likely an underestimate because it does not include boring sponges or encrusting sponges. The remineralization of organic matter by sponges could also serve as a mechanism for benthic-pelagic coupling, because sponges filter feed exclusively from the water column, yet the nutrients they expel are released near the benthos. The effect of increased nutrient levels on corals and other reef organisms is still controversial (see references in Chapter 1); however,

if elevated nutrients do damage reef health, then sponges may be a contributing factor to coral reef decline.

This study also revealed the predominant role of *X. muta* as a source of nitrate, a feature that could suggest a degree of instability in the ecological status quo. Because of its biomass dominance in the population, this sponge drives most of the measured benthic nitrate flux (Chapter 3). However, *X. muta* appears to be vulnerable to bleaching (Vicente 1990) and disease (Cowart et al. 2006). Therefore, if this one species were to suffer widespread mortality, it would dramatically alter rates of organic matter remineralization and benthic nitrification, a change that could have cascading effects for other reef taxa (Butler et al. 1995). This lack of ecological redundancy could therefore be regarded as a symptom of vulnerability.

Another facet of sponge N cycling revealed by this research was the apparent ubiquity of nitrification among HMA sponges. Recent work on sponge microbial communities suggests that these consortia may be more similar than was previously thought (Hentschel et al. 2006). Ammonia oxidation and nitrification, therefore, may be part of this characteristic microbial community. However, it is yet unknown whether the primary agents of ammonia oxidation are bacterial or archaeal. Because the only known nitrite oxidizers are bacterial, it is assumed that bacteria must play at least some role in sponge nitrification (although this could change if archaeal nitrite oxidizers are discovered).

The N_2 fixation rates measured in Chapter 6 were too anemic to produce very low natural abundance $\delta^{15}N$ values, which was a surprising result. Wilkinson and Fay's (1979) report on sponge N_2 fixation has often been cited in subsequent literature, even after Wilkinson later published data showing that their evidence was inconsistent (Wilkinson

1999). The association between N_2 fixers and sponges seemed logical because of the typically low nutrient concentrations in reef waters and the large quantities of N needed by sponges to generate their spongin skeletal material. However, N_2 fixation is an energetically costly process that is generally performed in zones of nitrogen deficit, and the large fluxes of DIN sponges with both high and low $\delta^{15}N$ values (Chapter 4) suggest that they may not be N limited. In retrospect, N_2 fixation alone is probably not capable of producing the lowest $\delta^{15}N$ values (some much less than 0‰), given that there is almost certainly concurrent consumption of organic matter, which will subsequently be enriched by ~ 3.4‰. That is not to say that these processes are not occurring; rather, they cannot be the primary explanation for the dichotomy in sponge $\delta^{15}N$ values. Furthermore, N_2 fixation in the water column is apparently quite rapid, reducing the potential ecological significance of sponge-hosted N_2 fixation. It had been hypothesized that the low-oxygen environment of sponge tissue could accelerate N_2 fixation; however, apparent fixation rates were commensurate with water column rates (adjusted for the higher microbial density in sponges). Therefore, the unique environment provided by the sponge does not appear to benefit N_2 fixers.

Speculation on "the $\delta^{15}N$ value dichotomy"

DIN assimilation

Perhaps the most compelling question generated by the stable isotope study was: What is causing the low $\delta^{15}N$ values in some HMA sponges? Unfortunately, none of the results generated have definitively answered that question. However, I have been able to eliminate some possibilities. As discussed above, the very low $\delta^{15}N$ values of some sponges suggest a contribution from a very light source, or a process with a very strong fractionation.

The source with the greatest potential for fractionation is assimilation of dissolved inorganic N by microbial symbionts. Assuming that photosymbionts (Chapter 3) and nitrifiers (Chapter 5) are actively fixing carbon, some incorporation of inorganic nitrogen is expected. Results from Chapter 5 and Appendix E suggested that metabolically produced nitrate is not retained, but that ammonium may be. Although low sponge $\delta^{15}N$ values resulting from ammonium uptake does not neatly fit all the data, there is some evidence to support it: 1) species with low $\delta^{15}N$ values containing microalgae have spatial gradients in $\delta^{15}N$ values that are qualitatively consistent with increased DIN uptake with increased light (Chapter 3), 2) the mass-specific DIN flux of HMA species is significantly lower than LMA species (but only when the genus *Ircinia* is excluded) (Chapter 4), 3) the ectosome $\delta^{15}N$ value of *A. cauliformis* is ~ 0.5‰ lower than its endosome (Chapter 3), and 4) data from Bayside sponges (Appendix D and Behringer and Butler 2006) show that HMA sponges in Florida Bay can have extremely depleted $\delta^{15}N$ values, even when they are located in areas classified as "impacted" (Behringer and Butler 2006). Sediment and SPOM $\delta^{15}N$ values from bayside sites suggest that the particulate nitrogen there is more enriched in ^{15}N compared to oceanside sites (Chapter 2), but the nutrient-replete environment could cause increased partitioning of isotopes.

The main problem with the ammonium uptake hypothesis is that not all HMA sponges have low $\delta^{15}N$ values, and there is no obvious difference between low and high $\delta^{15}N$ HMA sponges that can explain this fact. Potential explanations include 1) less DIN uptake occurs in some species (perhaps due to lower density of microbes) and 2) additional processes influence the isotopic composition in some species. The first possibility cannot be evaluated without knowing the microbial cell densities in these species, and this analysis was

not performed. However, it is reasonable to expect some interspecies variability in the ratio of sponge cells to microbial cells. The pigment data do not reveal any correlations between photosymbiont abundance and $\delta^{15}N$ values among HMA sponges. However, heterotrophic and chemoautotrophic organisms may also assimilate significant amounts of ammonium; therefore a measure of overall microbial cell density is necessary to evaluate the potential effect of symbiont density on isotopic fractionation during ammonium assimilation.

Additional processes, especially those that partially consume the ammonium substrate, could affect sponge $\delta^{15}N$ values in a couple of ways. First, fractionation of N isotopes during ammonium assimilation is expected to be greatest when concentrations are high. Therefore, additional demands on a finite pool of ammonium will likely reduce the apparent fractionation during uptake: if all available ammonium is assimilated, then there will be zero fractionation. Competing processes could further affect sponge isotopic composition if such processes preferentially select ^{14}N, and the products are not assimilated into biomass. For example, nitrification occurs with a large fractionation, leaving the remaining ammonium enriched in ^{15}N (i.e. Rayleigh distillation), and the resulting nitrate is not apparently assimilated by sponge microbes (Chapter 5, Appendix E). Rayleigh distillation was observed in the $\delta^{15}N$ values of the nitrate expelled by sponges (Chapter 5), so it is logical to assume that it also affects the remaining pool of ammonium. Therefore, the relative rates of nitrification to assimilation could exert a measure of control on sponge $\delta^{15}N$ values.

However, all HMA sponges studied were found to host nitrification, including those with both low and high $\delta^{15}N$ values. Therefore, some additional factor must determine the net isotope effect of the two processes. If the ammonium substrate is limiting (and several

species effectively do not release ammonium (Chapter 4)), then microbes assimilating ammonium could be in competition with ammonia oxidizing organisms. The spatial arrangement of microbial communities could therefore be a factor in such a competition because the advection of water through the sponge tissue is generally unidirectional. If the ammonium pool is partially nitrified "upstream" then ^{15}N enriched ammonium will be delivered to other cells "downstream" where it could be assimilated, producing biomass with high $\delta^{15}N$ values. If assimilation occurs "upstream", then isotopically "light" N will be incorporated into biomass, and isotopically "heavy" ammonium will be nitrified "downstream" and released. A simple 2 step Rayleigh distillation model shows the effect of order on the isotopic composition of assimilated, oxidized, and residual ammonium (Appendix F). The order in which assimilation and nitrification processes occur could therefore be as important (or more important) than their relative rates.

This is, of course, an oversimplification because heterotrophic activity likely produces ammonium throughout the sponge interior. Rather, this discussion is simply intended to demonstrate the potential effects of competing processes. Further, it is uncertain whether microbial communities could be spatially separated (e.g. "upstream" and "downstream" areas), although some limitations may exist because of physical and chemical factors. For example, photosymbionts are likely restricted to the outer layer in order to have access to light, and nitrifiers may be similarly restricted in order to have access to oxygen (Chapter 6). Heterotrophic organisms may not have such restrictions, especially if they are capable of anaerobic respiration. Therefore, the relative importance of assimilation versus oxidation and the order in which the two occur could be influenced by the ratio of surface area to volume, and oxygen concentrations in the sponge tissue. Morphology and pumping

rate may therefore be important factors in determining the overall $\delta^{15}N$ value of sponge tissue.

The relative rates of oxidation versus assimilation (by phototrophs, heterotrophs and chemoautotrophs) and their effects on N isotope fractionation are therefore very difficult to predict. Although I have direct measurements of nitrification for 8 species, I unfortunately have no reliable direct measurements of ammonium assimilation. The few preliminary data that I obtained are not consistent with assimilation of ammonium by HMA sponges (Appendix E), even though other evidence suggests that some assimilation is occurring (Chapter 5). However, the assimilation data are very limited, and could easily have been compromised due to adsorption of ammonium (See Appendix E for more details).

Gaseous N production

Biogeochemical processes that produce a gaseous loss of N could also affect the $\delta^{15}N$ value of the residual DIN pool, and a stoichiometric study by Martens et al. (in prep) revealed a possible imbalance in the N budget of *X. muta* that is consistent with gaseous N production. There are several processes that can couple with ammonia oxidation and/or nitrification to produce gaseous N compounds, including denitrification, anammox, N_2O production (during nitrification or denitrification), and nitrifier denitrification. None of these processes have yet been reported in sponges, but the hypoxic, nitrate-rich internal sponge tissue would provide a suitable environment for them to occur. Furthermore, N_2O production is a common byproduct of nitrification when oxygen concentrations are low (Goreau et al. 1980).

My research on nitrification does provide some constraints on potential rates of gaseous N loss. For example, all of the known processes that produce N_2 or N_2O require a source of oxidized N, so blocking nitrification should also block these processes. However, inhibiting ammonia oxidation did not significantly change the total flux of DIN from *A. cauliformis* or *S. aurea* (although there was a non-significant increase of about 10% for *A. cauliformis*). Therefore, gaseous N production, if it is occurring at all in these sponges, is likely occurring at low rates compared to DIN release.

Another constraint on potential rates of N_2 production comes from the isotopic composition of nitrate in sponge excurrent water, which ranged from -19.4 to 1.8‰ (after correction for ambient nitrate, Chapter 5). This is a broad range of values, yet even the highest are lower than those usually associated with denitrification in the water column (Sigman et al. 2000). However, the enrichment in the residual nitrate may have been masked by the strong fractionation associated with nitrification (Casciotti et al. 2003), depending on the rates of the two processes. Assuming a fractionation factor of 20‰ (Sutka et al. 2004), an enrichment of 3‰ (the difference between high and low $\delta^{15}N$ sponges) could be produced by consuming only ~10% of the available nitrate. If the N_2 production proceeds with a lower fractionation factor (as in sediments; Brandes and Devol 1997) then the enrichment in $^{15}NO_3^-$ would be proportionally less.

It is therefore possible that certain species of HMA sponge are producing N_2 or N_2O, but without a large, detectable isotopic enrichment in the effluent nitrate or biomass. Because nitrification rates in sponges are so high (11 mmol m^{-2} d^{-1}), a small byproduct of this process could generate an environmentally significant flux of N_2 or N_2O. For example, if *X. muta* alone produces gaseous N at 10% of its nitrate release rate, this translates into roughly

40 μmol m^{-2} h^{-1} (as N) of N_2 or N_2O. This is an order of magnitude higher than the 3 μmol m^{-2} h^{-1} measured in coral reef sediments (Miyajima et al. 2001), despite the fact that the sediment rate assumes 100% coverage, whereas the *X. muta* estimate does not.

Preliminary data

In 2006, some preliminary experiments were conducted to test for the production of N_2 in 6 species (methods and results in Appendix F). *I. felix* is the only sponge that showed any evidence of N_2 production, and at potential rates that are low compared to other benthic rates. Although 6 species were tested for N_2 production, it is possible that the group most likely to host this process was excluded. The higher $\delta^{15}N$ values of some other species (e.g., *X. muta*), make them likely candidates for gaseous N production. HMA sponges with high $\delta^{15}N$ values make up over 70% of the sponge biomass on Conch Reef (Chapter 3), and are therefore capable of significantly affecting the N cycling. However, none of these sponges were used because their large size makes them difficult subjects for incubation experiments. This greater size may also make them more suitable hosts for anaerobic processes, as the greater interior volume could accommodate larger anaerobic microbial communities. Furthermore, N_2O was not analyzed in these experiments, and so a potentially important part of the flux has not been quantified.

Future directions

Sponges have long been regarded as among the simplest animals. Although this may be true in the anatomical sense, it is absolutely false in the biogeochemical sense. Because of this complexity, studying biogeochemical processes in sponges has presented many unique

challenges. However, the magnitude of sponge DIN fluxes (Chapter 3) provides ample justification for attempting to resolve these challenges because elevated inorganic nitrogen levels can have negative consequences for coral reef ecology (Chapter 1). Furthermore, the phenomenon of nitrification in sponges has proved to be a very interesting subject, both in terms of its source and its potential byproducts. The provenance of sponge nitrate will likely be resolved as more becomes known about ammonia oxidizing archaea. The influence of ammonia oxidation on sponge isotopic composition will be more difficult to resolve, but the next steps to doing so are to measure microbial abundance in sponge tissue and to obtain reliable measurements of ammonium assimilation.

N_2 and N_2O production processes are critically important for balancing N_2 fixation (both natural and anthropogenic) and avoiding excessive buildup of fixed N in aquatic systems. Coral reefs require low nutrient levels to maintain ecological health, and so these processes may be especially important for that ecosystem. Furthermore, N_2O is a powerful greenhouse gas, and fluxes of N_2O from coral reef ecosystems to the atmosphere are not yet well understood. Given the high rates of nitrification by sponges, and the potential importance of gaseous N production, further investigation of this process is warranted. HMA species with high $\delta^{15}N$ values and low mass-specific DIN flux rates are logical choices for experimental subjects.

Coral reefs are generally considered oligotrophic environments. However, the results from this research provide an image of HMA sponge interiors very different from the medium that surrounds them. Sponges appear to be a nutrient-rich, hypoxic, soup where oxygen concentrations largely determine the mode of metabolism, an image that perhaps recalls the Precambrian world in which sponges first evolved. An example of how life can

be primitive yet complex, sponges will undoubtedly continue to intrigue scientists, and sponge research will continue to reveal new insights into their hidden interior world.

Tables

Species	Type	Nitrification	$\delta^{15}N$	Photosymbionts	O_2 profile	N2 fixation	N_2 production
A. cauliformis	HMA	Yes	2.0 ± 0.2	Yes	Hypoxic	Limited	Undetectable
A. fistularis	HMA	Yes		Yes			Undetectable
A. archerii	HMA	Yes		Yes	Hypoxic		
A. conifera	HMA	Yes	4.5 ± 0.1	No	Hypoxic		
A. compressa	LMA	No	4.76 ± 0.46	No			Undetectable
A. lacunosa	HMA	Yes		Yes			
C. nucula*	HMA	Yes					
C. plicifera	LMA						
C. vaginalis	LMA	No	3.7 ± 0.1	No			Undetectable
I. birotulata	LMA		3.82 ± 0.26	No			
I. campana	HMA	Yes	-0.1 ± 0.6	Yes	Hypoxic	Limited	
I. felix	HMA	Yes	1.2 ± 0.2	Yes	Hypoxic		Limited
I. strobilina	HMA	Yes	1.6 ± 0.71	No	Hypoxic		
I. variabilis*	HMA		-0.02 ± 0.39				
N. digitalis	LMA	No	4.2 ± 0.1	No	Oxic		
N. erecta	LMA	No	4.3 ± 0.1	No	Oxic	Very limited	Undetectable
S. aurea	HMA	Yes	2.3 ± 0.3	No			Undetectable
P. crassa	HMA	Yes		Yes			
V. rigidia	HMA			Yes			
X. muta	HMA	Yes	4.55 ± 0.43	Yes	Oxic/ variable		

Table 7.1. Summary of analysis performed on various sponges.

Figures

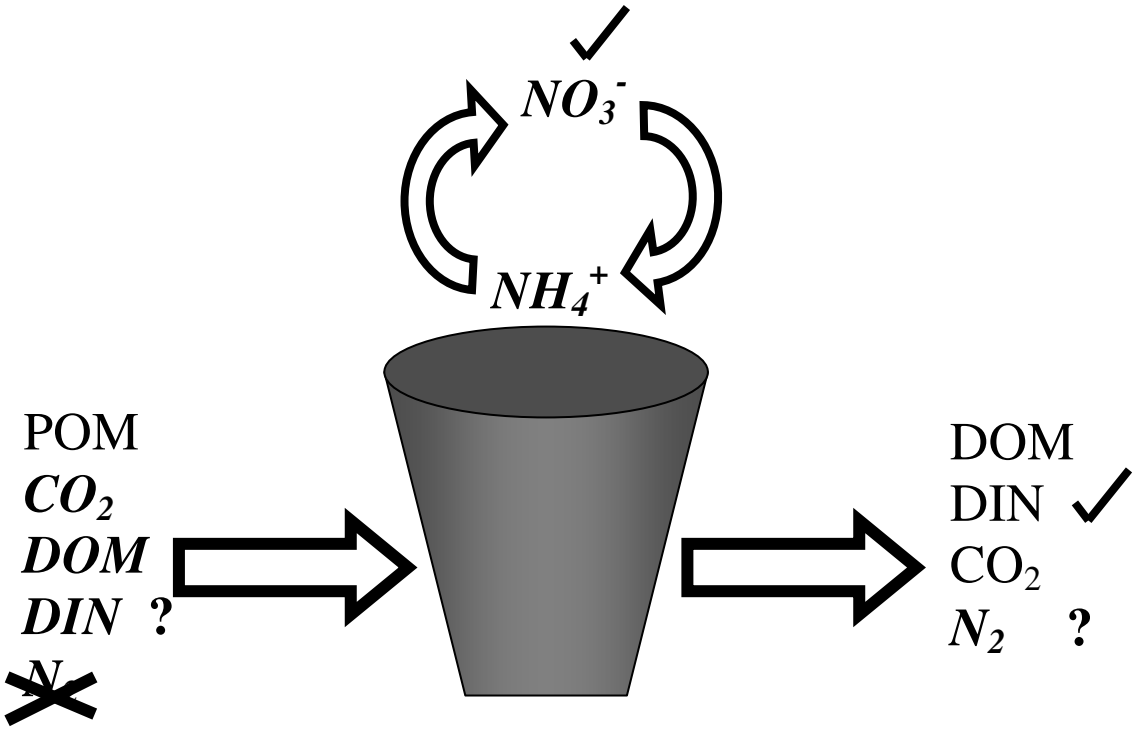

Figure 7.1. Summary of biogeochemical processes potentially performed by HMA sponges. Processes found not to occur at significant rates are marked with an X. Processes found to occur at significant rates are marked with a check.

APPENDIX A

C and N stable isotopic composition of particulate organic matter and sponges shown in GIS figures (Chapters 2 and 3)

		C/N, mol/mol)		δ^{15}N, ‰		δ^{13}C, ‰	
Site	**N**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**
Alligator Bay	1	10.62	.	4.65	.	-17.37	.
Conch Deep	2	8.31	0.19	2.51	0.11	-16.94	0.08
Cotton Key	3	8.89	0.20	2.60	0.05	-13.62	0.35
Cowpen Cut	3	9.87	0.17	3.57	0.15	-13.94	0.45
Habitat	2	8.61	0.05	2.33	0.21	-16.53	0.38
Little Buttonwood Sound	3	10.53	0.45	4.44	0.18	-17.21	0.44
Nest Key	3	10.77	1.80	5.63	0.44	-13.47	0.09
Pickles	4	8.94	0.48	1.64	0.49	-15.25	0.19
Shell Key	3	12.16	0.19	4.30	1.06	-13.79	0.61
Swash Key	3	11.27	0.21	5.14	0.22	-13.11	0.30
Tavernier Rocks	4	9.27	0.22	1.38	0.48	-13.74	0.22
Tern Key	2	10.27	0.09	5.43	0.34	-17.12	1.07
Three Sisters	6	9.24	0.19	1.04	0.09	-13.79	0.18
Triangles	3	9.35	0.60	1.25	0.30	-14.04	0.75

Table A.1. Site means and standard deviations for sediment analysis.

Site	N	C/N, mol/mol		$\delta^{13}C$, ‰		$\delta^{15}N$, ‰	
		Average	SD	Average	SD	Average	SD
Bayside	6	7.67	0.22	-19.92	2.27	4.16	1.24
Creek	2	10.04	1.28	-18.38	0.37	2.79	0.70
Ocean	5	8.21	.	-21.86	1.02	1.99	0.44
Three Sisters	5	9.82	0.51	-15.07	0.51	1.06	0.23

Table A.2. Site means and standard deviations for Suspended Particulate Organic Matter, grouped by Site Class. Three Sisters excluded because resuspension of sediments.

Sponges

Species	$\delta^{15}N$, ‰	$\delta^{13}C$, ‰	SD $\delta^{15}N$	SD $\delta^{13}C$	N
Oceanside					
Agelas clathrodes	5.09	-17.68	.	.	1
Agelas conifera	4.45	-18.22	0.04	0.16	2
Agelas schmidti	4.40	-17.51	0.53	0.38	9
Amphimedon compressa	4.76	-17.21	0.46	1.58	24
Aplysina cauliformis	1.94	-17.89	0.74	0.82	34
Aplysina fulva	2.25	-18.26	0.23	0.20	3
Callyspongia plicifera	4.01	-18.24	0.35	0.13	2
Callyspongia vaginalis	3.61	-16.43	0.37	1.90	32
Calyx podatypa	3.20	-19.51	.	.	1
Cribrochalina vasculum	3.75	-18.97	.	.	1
Ectyoplasia ferox	2.28	-19.19	1.24	0.23	3
Iotrochota birotulata	3.82	-18.38	0.26	0.24	14
Ircinia campana	0.06	-15.77	1.48	0.53	14
Ircinia felix	1.18	-16.10	0.93	0.92	30
Ircinia strobilina	1.60	-15.80	0.71	1.22	26
Mycale laevis	4.04	-17.51	.	.	1
Mycale laxissima	4.63	-13.61	0.70	1.35	8
Niphates digitalis	4.20	-16.65	0.45	1.29	25
Niphates erecta	4.25	-16.19	0.45	1.76	39
Ptilocaulis sp.	4.59	-19.52	0.20	0.15	3
Smenospongia aurea	2.25	-16.57	0.54	0.89	4
Spheciospongia vesparium ocean	4.33	-14.55	0.05	0.52	2
Ulosa ruetzleri	5.77	-19.48	0.16	0.12	3
Verongula gigantea	3.48	-17.97	0.21	0.21	2
Xestospongia muta	4.55	-19.48	0.43	1.05	21
Bayside					
Chondrilla nucula	5.23	-14.52	0.87	2.05	24
Ircinia variablis	-0.02	-12.31	0.39	1.93	20
Psuedo Loggerhead	1.39	-13.66	0.50	0.50	2
Spheciospongia vesparium	4.46	-13.47	3.27	2.23	15
Spongia officinalis	-1.63	-12.11	0.04	2.06	2
Tedania Ignis	6.33	-17.48	0.27	0.17	6

Table A.3. Species averages of $\delta^{13}C$ and $\delta^{15}N$ values.

Species	Site	N	$\delta^{13}C$, ‰		$\delta^{15}N$, ‰	
			Average	SD	Average	SD
Amphimedon compressa	Duane	2	-19.10	0.08	4.04	0.45
Amphimedon compressa	Conch-Deep	3	-19.18	0.20	4.61	0.16
Amphimedon compressa	Conch-Mid	3	-18.31	0.08	4.67	0.28
Amphimedon compressa	Pickles	3	-17.90	0.25	4.68	0.30
Amphimedon compressa	Conch-Shallow	7	-17.00	0.41	4.74	0.51
Amphimedon compressa	Three Sisters	2	-15.49	0.29	5.04	0.55
Amphimedon compressa	Tavernier Rocks	2	-14.30	0.49	5.42	0.05
Amphimedon compressa	Triangles	2	-15.09	0.32	5.06	0.08
Aplysina cauliformis	Duane	8	-18.40	0.23	2.20	0.66
Aplysina cauliformis	Conch-Deep	5	-18.09	0.27	2.90	0.11
Aplysina cauliformis	Conch-Mid	4	-18.37	0.67	1.95	0.45
Aplysina cauliformis	Pickles	2	-18.72	0.07	1.61	0.21
Aplysina cauliformis	Conch-Shallow	2	-18.02	0.53	2.10	0.65
Aplysina cauliformis	Three Sisters	8	-17.35	0.79	1.79	0.43
Aplysina cauliformis	Tavernier Rocks	2	-15.93	0.25	1.45	0.52
Aplysina cauliformis	Triangles	3	-17.68	0.59	0.52	0.49
Callyspongia vaginalis	Duane	8	-18.46	0.24	3.48	0.26
Callyspongia vaginalis	Conch-Deep	3	-18.45	0.08	3.61	0.23
Callyspongia vaginalis	Conch-Mid	2	-18.15	0.34	3.67	0.10
Callyspongia vaginalis	Pickles	3	-17.04	0.16	3.72	0.53
Callyspongia vaginalis	Conch-Shallow	3	-16.07	0.17	3.48	0.50
Callyspongia vaginalis	Three Sisters	5	-14.83	0.34	3.65	0.54
Callyspongia vaginalis	Tavernier Rocks	3	-13.55	0.26	3.81	0.50
Callyspongia vaginalis	Triangles	3	-14.46	0.24	3.58	0.45
Ircinia felix	Duane	1	-16.47	.	1.20	.
Ircinia felix	Conch-Deep	2	-17.11	0.48	1.54	0.74
Ircinia felix	Conch-Mid	5	-17.00	0.23	2.43	0.41
Ircinia felix	Pickles	3	-16.95	0.34	1.75	0.30
Ircinia felix	Conch-Shallow	4	-16.58	0.45	1.31	0.51
Ircinia felix	Three Sisters	6	-15.21	0.76	0.63	0.55
Ircinia felix	Tavernier Rocks	2	-14.84	0.04	-0.41	0.99
Ircinia felix	Triangles	3	-15.94	0.25	0.44	0.89
Ircinia strobilina	Duane	7	-16.76	0.55	1.97	0.46
Ircinia strobilina	Conch-Mid	2	-15.99	0.24	2.19	0.24
Ircinia strobilina	Habitat	4	-16.55	0.22	0.41	0.65
Ircinia strobilina	Conch-Shallow	8	-15.82	0.77	1.49	0.51
Ircinia strobilina	Three Sisters	3	-13.96	0.31	1.99	0.13
Ircinia strobilina	Triangles	2	-13.38	0.12	1.89	0.10
Niphates erecta	Duane	8	-18.70	1.05	4.02	0.51
Niphates erecta	Conch-Deep	3	-17.29	0.35	4.13	0.07
Niphates erecta	Conch-Mid	3	-17.21	0.69	4.44	0.38
Niphates erecta	Pickles	5	-16.53	0.12	4.52	0.25
Niphates erecta	Conch-Shallow	5	-15.84	0.22	3.70	0.46

Niphates erecta	Three Sisters	10	-14.73	0.32	4.44	0.34
Niphates erecta	Tavernier Rocks	2	-13.41	0.24	4.73	0.44
Niphates erecta	Triangles	2	-14.14	0.10	4.34	0.00

Table A.4. Species-site means of isotopic compositions of sponges used in spatial analysis.

APPENDIX B

Regression statistics for sponges in spatial analysis

			$\delta^{13}C$, ‰			$\delta^{15}N$, ‰		
Factor	**Species**	**Type**	**Slope**	**p**	**R^2**	**Slope**	**p**	**R^2**
depth	*Aplysina cauliformis*	HMA	-0.03	0.22	0.24	0.04	0.04	0.54
distance to 20m	*Aplysina cauliformis*	HMA	0.26	0.02	0.60	-0.20	0.03	0.58
distance to shore	*Aplysina cauliformis*	HMA	-0.26	0.00	0.78	0.12	0.17	0.29
depth	*Ircinia felix*	HMA	-0.04	0.14	0.33	0.03	0.23	0.23
distance to 20m	*Ircinia felix*	HMA	0.26	0.02	0.61	-0.27	0.02	0.65
distance to shore	*Ircinia felix*	HMA	-0.21	0.04	0.54	0.22	0.03	0.56
depth	*Ircinia strobilina*	HMA	-0.08	0.03	0.73	-0.02	0.36	0.21
distance to 20m	*Ircinia strobilina*	HMA	0.53	0.00	0.93	0.05	0.70	0.04
distance to shore	*Ircinia strobilina*	HMA	-0.43	0.03	0.74	-0.11	0.36	0.21
depth	*Amphimedon compressa*	LMA	-0.13	0.01	0.75	-0.03	0.01	0.71
distance to 20m	*Amphimedon compressa*	LMA	0.69	0.00	0.92	0.14	0.00	0.81
distance to shore	*Amphimedon compressa*	LMA	-0.56	0.00	0.81	-0.13	0.00	0.92
depth	*Callyspongia vaginalis*	LMA	-0.14	0.01	0.75	0.00	0.30	0.17
distance to 20m	*Callyspongia vaginalis*	LMA	0.70	0.00	0.90	0.02	0.32	0.16
distance to shore	*Callyspongia vaginalis*	LMA	-0.58	0.00	0.80	-0.02	0.13	0.34
depth	*Niphates erecta*	LMA	-0.13	0.00	0.79	-0.01	0.33	0.16
distance to 20m	*Niphates erecta*	LMA	0.66	0.00	0.94	0.06	0.25	0.21
distance to shore	*Niphates erecta*	LMA	-0.56	0.00	0.89	-0.05	0.21	0.25

Table B.1. Statistical parameters of 3 LMA species regressed with respect to depth, distance to shore, and distance to 20 m isobath.

	C/N, mol/mol			$\delta^{13}C$, ‰			$\delta^{15}N$, ‰		
Term	**Slope**	**p**	**R^2**	**Slope**	**p**	**R^2**	**Slope**	**p**	**R^2**
depth	-0.04	0.00	0.97	-0.13	0.01	0.88	0.05	0.01	0.89
distance to 20m	0.14	0.02	0.49	0.47	0.02	0.56	-0.18	0.07	0.34
distance to shore	-0.10	0.12	0.76	-0.38	0.09	0.77	0.12	0.23	0.61

Table B.2. Results of linear regression of sediment $\delta^{13}C$ and $\delta^{15}N$ values on depth, distance to shore, and distance to 20 m isobath.

APPENDIX C

Water content of sponge tissue

Species	Site	Net Wet Wt. (g)	Net Dry Wt. (g)	% water
C. vaginalis	Tavernier Rocks	2.93	0.3	90
C. vaginalis	Duane	3.56	0.35	90
C. vaginalis	Triangles	3.53	0.34	90
C. vaginalis	Tavernier Rocks	3.68	0.38	90
C. vaginalis	Conch shallow	3.4	0.37	89
C. vaginalis	Tavernier Rocks	3.98	0.38	90
C. vaginalis	Pickles	4.3	0.43	90
C. vaginalis	Marker 43	4.23	0.51	88
C. vaginalis	Triangles	4.64	0.52	89
C. vaginalis	Triangles	4.87	0.53	89
C. vaginalis	Conch shallow	4.8	0.33	93
C. vaginalis	Marker 41	5.44	0.56	90
C. vaginalis	Conch shallow	5.59	0.58	90
C. vaginalis	Duane	6.21	0.56	91
C. vaginalis	Marker 42	6.16	0.59	90
C. vaginalis	Three sisters	6.97	0.81	88
C. vaginalis	Three sisters	7.33	0.85	88
C. vaginalis	Pickles	7.6	0.84	89
C. vaginalis	Conch	8.73	0.84	90
C. vaginalis	Conch mid	9.37	0.97	90
C. vaginalis	Pickles	9.19	1.17	87
C. vaginalis	Conch mid	10.91	0.95	91
C. vaginalis	Conch mid	12.72	1.57	88
C. vaginalis	Conch	13.09	1.26	90
C. vaginalis	Conch	14.29	1.48	90
C. vaginalis	Three sisters	14.75	1.54	90
I. strobilina	Conch shallow	2.1	0.45	79
I. strobilina	Conch shallow	1.8	0.08	96
I. strobilina	Conch mid	3.48	0.44	87
I. strobilina	Conch mid	2.3	0.4	83
I. strobilina	Triangles	4.46	0.87	80
I. strobilina	Triangles	1.58	0.31	80
I. strobilina	Duane	6.31	1.08	83
N. erecta	Three sisters	14.79	1.43	90
N. erecta	Three sisters	5.67	0.58	90
N. erecta	Triangles	2.94	0.34	88
N. erecta	Triangles	2.69	0.28	90
N. erecta	Pickles	9.29	1.1	88
N. erecta	Pickles	15.5	2.63	83

N. erecta	Tavernier Rocks	6.04	0.77	87
N. erecta	Tavernier Rocks	7.03	0.99	86
N. erecta	Marker 39	2.8	0.28	90
N. erecta	Duane	16.42	4.89	70
N. erecta	Duane	5.57	0.67	88
X. muta	Conch shallow	10.72	1.77	83
X. muta	Conch mid	10.61	1.64	85
X. muta	Conch mid	9.94	1.23	88
X. muta	Pickles	7.93	1.05	87
X. muta	Pickles	24.66	4.81	80
A. compressa	Tavernier Rocks	5.88	0.75	87
A. compressa	Tavernier Rocks	3.06	0.4	87
A. compressa	Triangles	3.53	0.42	88
A. compressa	Triangles	6.76	1.03	85
Average				**88**
Standard Deviation				**4**

Table C.1. Water content of sponge tissue calculated from wet and dry weights of sponge tissue.

APPENDIX D

C and N stable isotopic compositions of sponges collected from Florida Bay

Species	Site	N	$\delta^{15}N$, ‰		$\delta^{13}C$, ‰	
			Average	SD	Average	SD
Chondrilla nucula	Cowpen Ch.	6	4.51	0.55	-11.54	0.79
Chondrilla nucula	East of Porjoe Key	2	5.76	1.41	-15.41	0.45
Chondrilla nucula	Grouper Cr.	13	5.49	0.87	-15.84	0.83
Chondrilla nucula	Jewfish Creek	2	5.42	0.30	-15.28	0.54
Ircinia variablis	Cowpen Ch.	15	0.04	0.37	-11.29	0.61
Ircinia variablis	Jewfish Creek	3	-0.51	0.10	-15.68	1.04
Spheciospongia vesparium	Cowpen Ch.	3	6.56	0.11	-9.35	0.58
Spheciospongia vesparium	East of Porjoe Key	2	9.11	0.12	-13.41	0.43
Spheciospongia vesparium	Grouper Cr.	7	1.20	0.41	-14.82	0.55
Spheciospongia vesparium	Jewfish Creek	3	6.86	0.18	-14.51	0.31
Spongia officinalis	Cotton Key	2	-1.63	0.04	-12.11	2.06
Tedania Ignis	Grouper Cr.	6	6.33	0.27	-17.48	0.17

Table D.1. Species-site means and standard deviations for sponges collected in Florida Bay

APPENDIX E

Ammonium uptake experiment

Methods

Sponges from Three Sisters Reef were attached to PVC plates using methods described in Chapter 4. Each individual was cut into 5 pieces, with each piece attached to a separate plate and left on the reef to recover. After 2 weeks, the sponges were brought back to the lab and incubated with 0.2 μmM $^{15}NH_4^+$ or $^{15}NO_3^-$(approximately 10-20% of ambient levels) in filtered seawater (0.2 μm filter). Each incubation container held five sponge pieces from a given individual, and one piece was removed at each time point. The pieces were then frozen and N isotopic composition was later analyzed using methods described in Chapter 3.

Results and Discussion

Ammonium uptake

All sponge species acquired ammonium tracer over time (Figure 1). However, *A. compressa* appears to have incorporated more tracer than *N. erecta* or *A. cauliformis*. This is surprising because *A. cauliformis* is an HMA sponge, whereas the other two species are LMA sponges. Therefore it was expected that *A. cauliformis* would acquire the most ammonium tracer due to uptake by microbial symbionts. Some increase in $\delta^{15}N$ value was expected in LMA species due to 1) adsorption of ammonium and 2) uptake by microbial cells (presumably at lower density than HMA species). However, the greater rate of uptake by *A.*

compressa was unexpected. One possible explanation is that ammonia oxidizing symbionts in *A. cauliformis* converted some of the tracer to nitrate, which is not as readily assimilated by sponge microbies (Figure 2) and would not adsorb to organic matter as ammonium would. Therefore, this result may not be an accurate measurement of ammonium assimilation by these species.

Nitrate uptake

Nitrate incorporation was undetectable in the sponges studied (Figure 2).

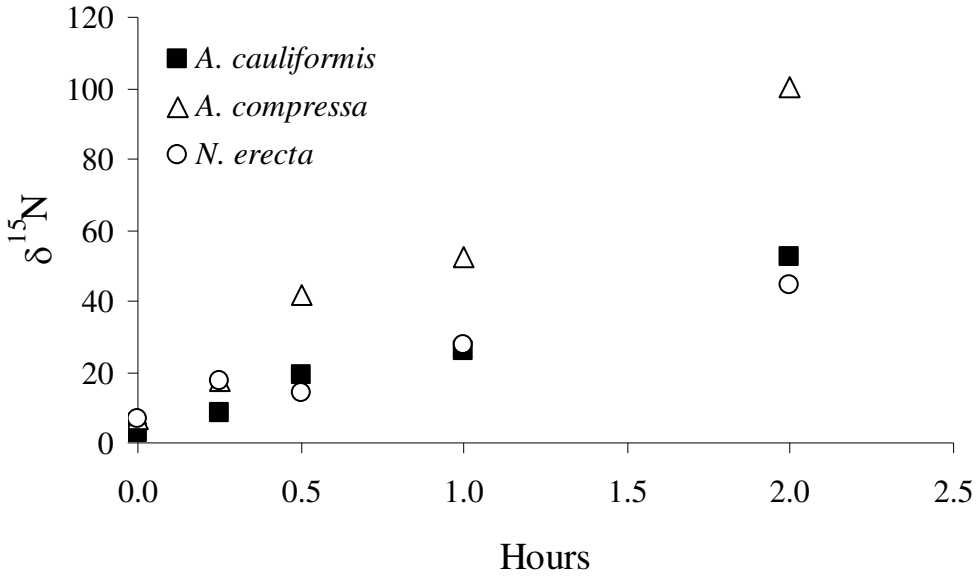

Figure E.1. $\delta^{15}N$ values of sponge tissue during incubation with labeled $^{15}NH_4^+$ tracer.

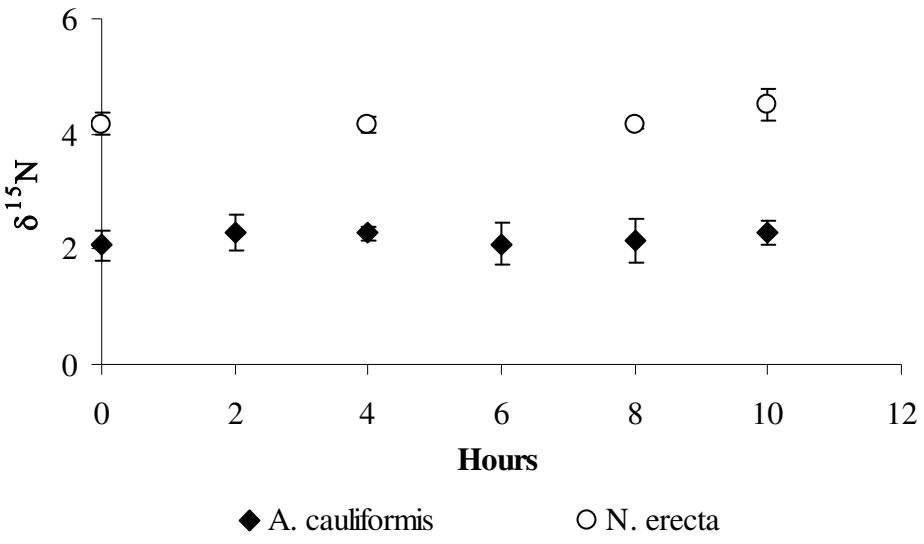

Figure E.2. $\delta^{15}N$ values of sponge tissue during incubation with labeled $^{15}NO_3^-$ tracer.

APPENDIX F

N_2 production experiments

Methods

I performed two sets of isotope tracer experiments to directly test for the presence of N_2 production in 4 HMA species and 2 LMA species. The first set was performed in May of 2006 and used only $^{15}NO_3^-$ as a tracer. The second set was performed in August of 2006 and used $^{15}NO_3^-$ and $^{15}NH_4^+$ both separately and in combination. Sponges were incubated in gas-tight containers (3.75 l) with unfiltered seawater, placed in a seawater bath to maintain constant temperature. ^{15}N labeled nitrate and/or ammonium were added to a final concentration of 0.2 μM, approximately 20% of the starting ambient value. The chambers were fitted with small powerhead pumps to circulate the water, two sample ports, and a thin butyl rubber septum. The rubber septum allowed us to monitor oxygen in the chambers using a Unisense Clark-style needle electrode and picoameter. Pure oxygen was added to chambers when oxygen concentrations decreased below 80%. Control experiments with no sponge were also performed in parallel. Water samples were collected with syringes at time points over a period of 24 hours and injected into evacuated crimp-seal vials fitted with butyl rubber stoppers. The samples were killed with 0.2 ml of 10M NaOH and stored submerged in water to minimize temperature fluctuation and reduce exposure to atmospheric N_2.

N_2 production samples. Before analysis, ~ 2 ml of He was exchanged for an equal volume of sample to create a headspace in the samples vials. The vials were shaken vigorously and

allowed to equilibrate. The ratios of ^{28}N, ^{29}N, and ^{30}N in 20 μL of headspace gas were then analyzed by GC-MS. Triplicate injections were made from each vial.

Results

Analysis of the vial headspace revealed that little of the ^{15}N labeled tracer was incorporated into the N_2 pool. In some cases, the isotopic composition did slightly increase with time (Figures 7-10) however in most cases the increase was not significant given the variability in the measurement and the seawater controls. The exception is *I. felix*, in which two out of three individuals increased by approximately 4 ‰ (Figure 4).

Disscussion

The only species that produced a significant change in the $\delta^{15}N$ of the dissolved N_2 pool was *I. felix* (2 of 3 individuals). The time course values of these two individuals are not linear, therefore, calculating a rate from these data is not possible. This variability is not entirely surprising, given that the large background of atmospheric N_2 makes it difficult to avoid contaminating samples and incubation chambers with atmospheric N_2 and diluting the tracer. Further experiments would have to be performed with this sponge in order to achieve a confident measure of N_2 production. However, for the sake of providing an estimate of denitrification potential, I will assume linearity from t=0 and calculate a rate based on the final inventory of new $^{29}N_2$ at the final time point. Using this approach, the two individuals of *I. felix* produced approximately 4 nmol N g^{-1} of N_2 over the 16 hour period or 6 nmol g^{-1} d^{-}

[1]. According the biomass survey (Chapter 3), there is approximately 40 mL m^{-2}, or 4 g m^{-2}, of *I. felix* on the reef, translating to 72 nmol N_2 g^{-1} m^{-2} d^{-1}. Even if all the sponges on the reef were actively denitrifying at this rate, it would still only produce about 3 μmol m^{-2} d^{-1}. This is an order of magnitude less than the rates in sediment and seagrass, which produce 20.4 - 24 μmol N m^{-2} d^{-1} and 32.4 - 42 μmol N m^{-2} d^{-1}, respectively, (Miyajima et al. 2001) and 4 orders of magnitude less than the DIN flux from the sponges themselves. These results therefore indicate that N_2 production is negligible, both in terms of reef N cycling rates and sponge metabolic rates.

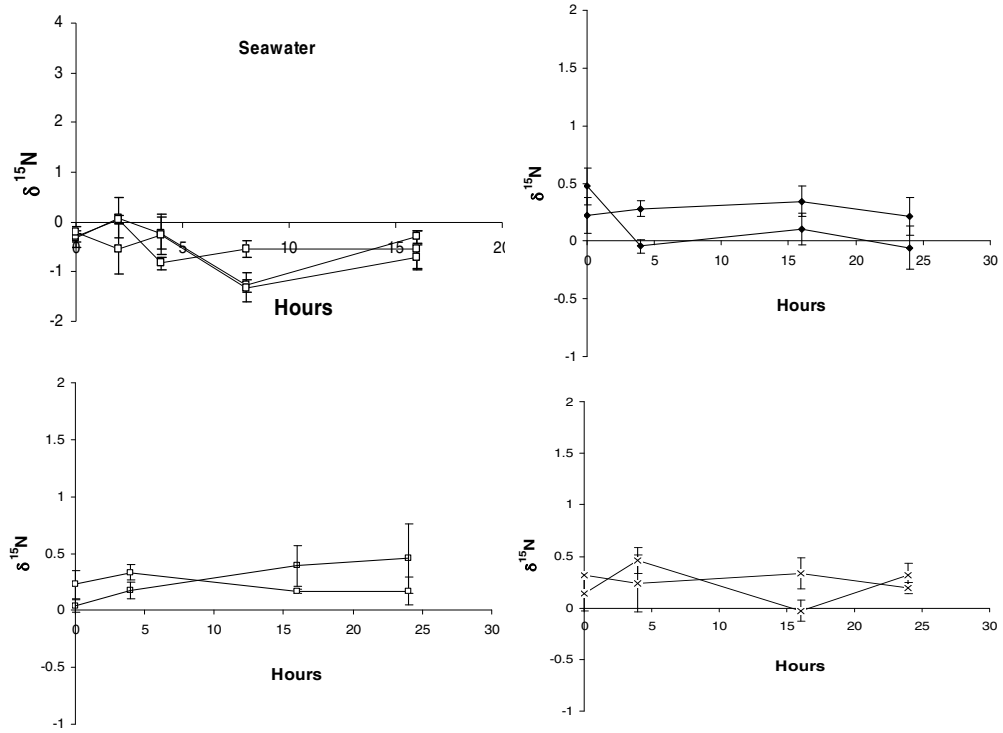

Figure F.1. $\delta^{15}N$ values of dissolved N_2 during incubations with seawater only. Incubations were ammended with $^{15}NO_3^-$ (open squares), $^{15}NH_4^+$ (closed diamonds) or both (X's).

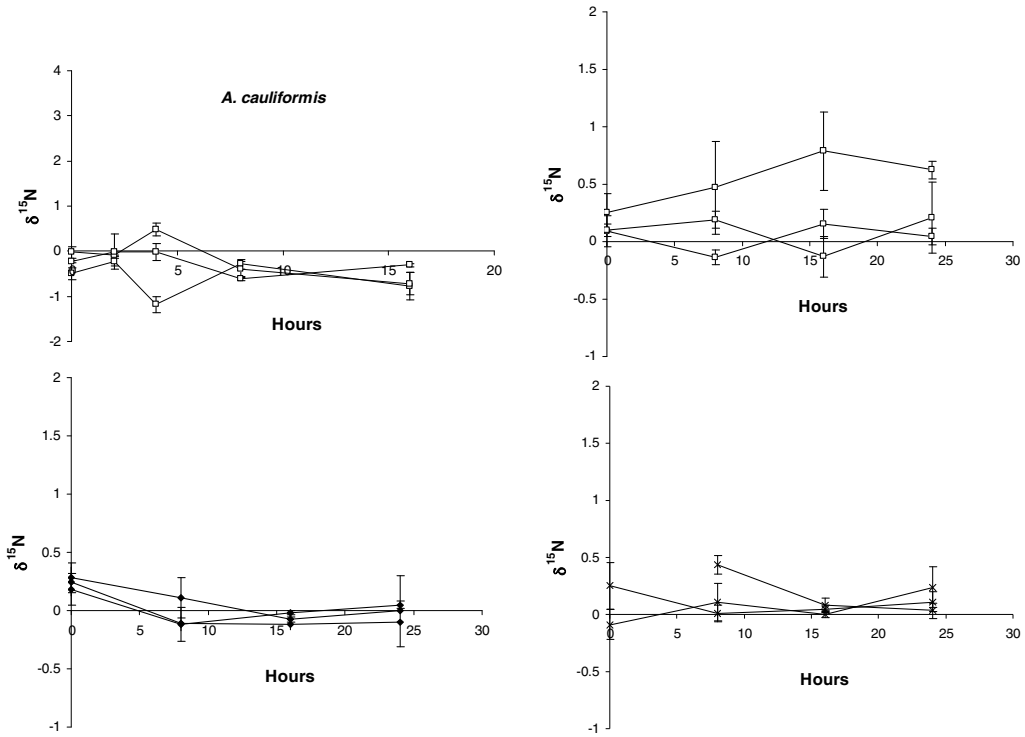

Figure F.2. $\delta^{15}N$ values of dissolved N_2 during incubations with *A. cauliformis*. Incubations were ammended with $^{15}NO_3^-$ (open squares), $^{15}NH_4^+$ (closed diamonds) or both (X's).

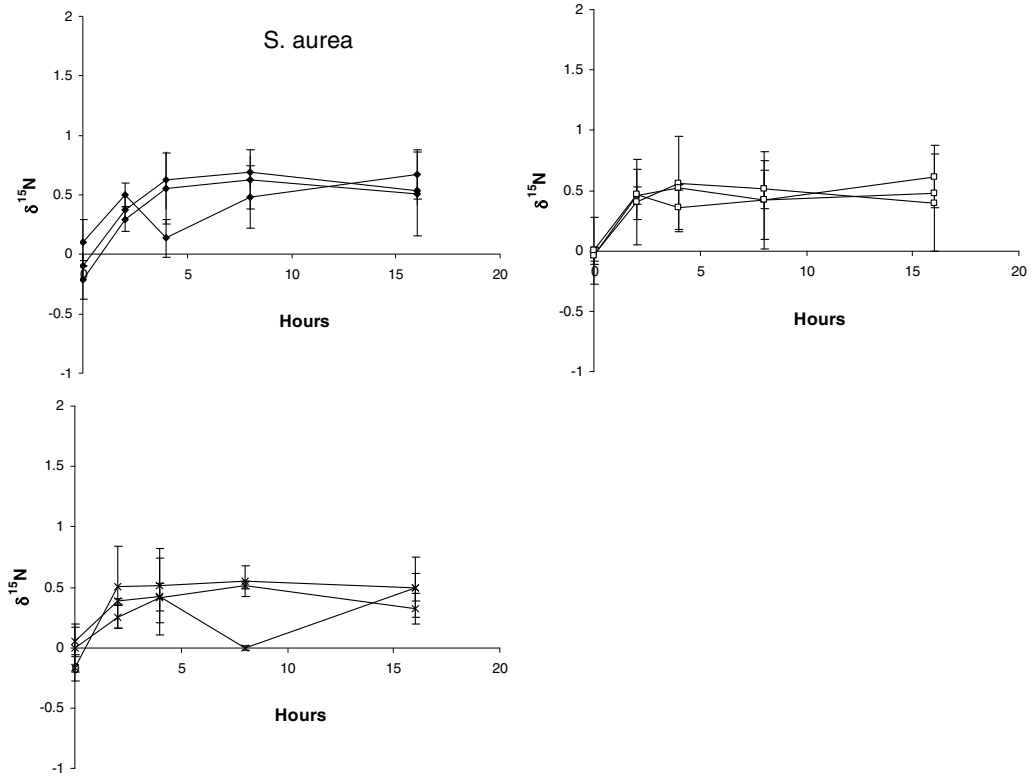

Figure F.3. $\delta^{15}N$ values of dissolved N_2 during incubation with *S. aurea*. Incubations were amended with $^{15}NO_3^-$ (open squares), $^{15}NH_4^+$ (closed diamonds) or both (X's).

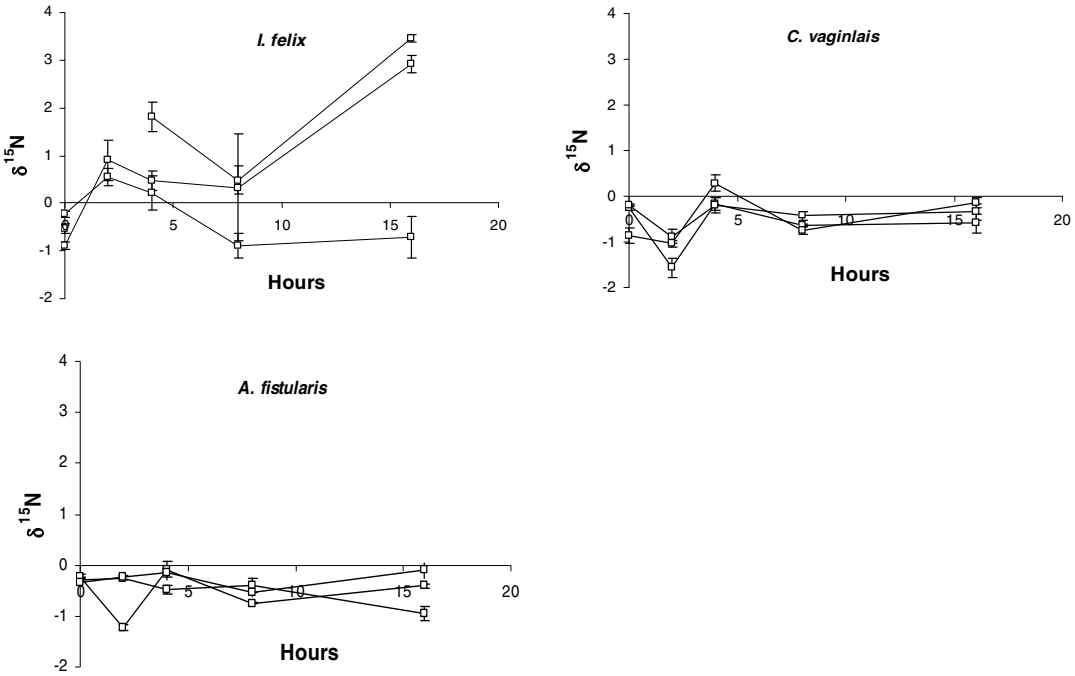

Figure F.4. $\delta^{15}N$ values of dissolved N_2 during incubation with *I. felix, C. vaginalis*, and *A. fistularis.* Incubations were amended with $^{15}NO_3^-$.

APPENDIX G

Potential effects of concurrent processes on isotopic composition of sponges

In order for inorganic nitrogen to influence the isotopic composition of sponge tissue, it must be incorporated into biomass. However, dissimilatory processes can also affect the resulting $\delta^{15}N$ values if assimilatory and dissimilatory processes consume the same substrate. In sponges, ammonium assimilation and nitrification are likely both occurring simultaneously. However, ammonia oxidizers and nitrifiers are obligate aerobes, and so they are restricted to oxic environments. Oxygen compounds decrease dramatically within mm of the tissue surface, and this feature could serve to concentrate nitrifying microorganisms in the surface tissue of the sponge. Therefore, a simple 2-step Rayleigh Distillation model was created in Excel to describe how the order in which nitrification and ammonium assimilation occur might affect the isotopic composition of assimilated ammonium. The model assumes a 10 ‰ fractionation factor for ammonium assimilation (Fogel and Cifuentes 1993) and a 20 ‰ fractionation factor for nitrification (Casciotti et al. 2003). In this model, the second reaction consumes 80% of the substrate not consumed by the first reaction, and the $\delta^{15}N$ value of the starting ammonium reactant is 3.5‰. The $\delta^{15}N$ of the resulting products are plotted against the fraction consumed by the first reaction.

Results

The order in which assimilation and nitrification occur had a large effect on the partitioning of isotopes in the model (Figures 1 and 2). Theoretical values were used in this model; therefore, the magnitude of the differences in $\delta^{15}N$ values for the different products is

somewhat hypothetical. However, the general trends remain the same regardless of fractionation factor. If ammonia oxidation occurs first, the ammonium subsequently assimilated becomes progressively enriched in ^{15}N. Therefore, location of assimilation and nitrification relative to the direction of water flow could influence the $\delta^{15}N$ value of assimilated ammonium.

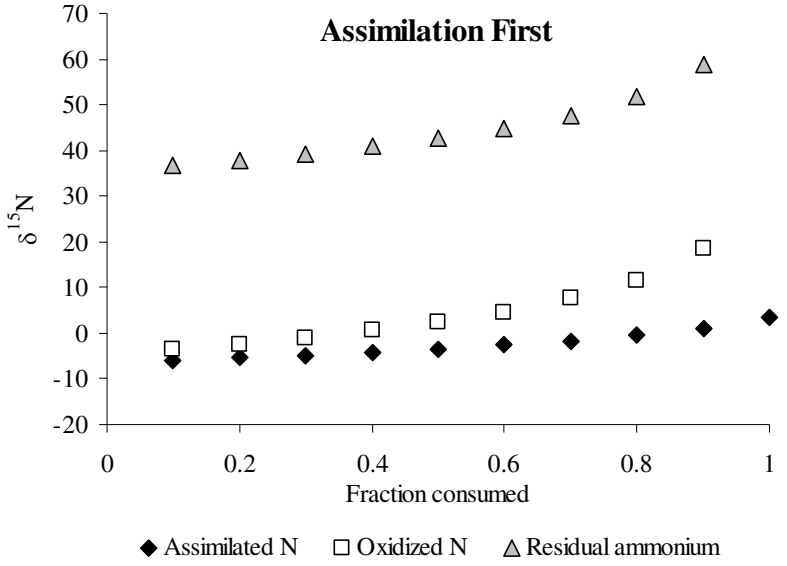

Figure G.1. $\delta^{15}N$ values of assimilated, oxidized, and residual ammonium modeled in a 2-step Rayleigh distillation, in which assimilation occurs first and ammonia oxidation occurs second.

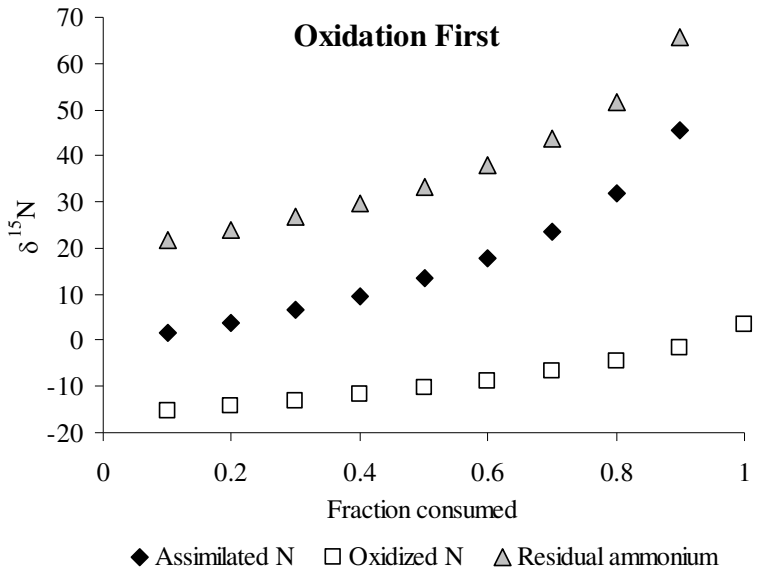

Figure G.2. $\delta^{15}N$ values of assimilated, oxidized, and residual ammonium modeled in a 2-step Rayleigh distillation, in which ammonia oxidation occurs first and assimilation occurs second.

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