STABLE ISOTOPE RATIOS OF BENTHIC MARINE FAUNA: DEVELOPING RECORDS OF ANTHROPOGENIC CHANGE

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STABLE ISOTOPE RATIOS OF BENTHIC MARINE FAUNA: DEVELOPING RECORDS OF ANTHROPOGENIC CHANGE

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Worldwide, the escalation of nutrient pollution from multiple sources is causing the collapse of coastal marine ecosystems. Anthropogenic inputs of nitrogen (N) from agriculture, fossil fuel and biomass burning, and urban development have ushered in an era of coastal eutrophication and consequently, widespread loss of biodiversity and ecosystem services. Often cited as the “canaries” of the sea, corals and the reefs they form are highly sensitive to this pollution, and the dramatic loss of coral reef habitat has coincided with human development. Yet, as this problem has only received attention in the last several decades, there is a critical need to understand how human-derived sources of pollution have changed since industrialization, and establish historical baselines, from which we can assess global change in today’s world.

This dissertation expands on our understanding of coastal pollution using isotope ratios of common tropical benthic biota. In chapter 1, I show that gorgonian corals are particularly useful recorders for environmental N sources, and report several considerations for sampling and data interpretation using in situ collections and lab experiments. In chapter 2, I use common benthic calcareous algae to quantify spatial and intra-annual variability in the Florida Keys. Using a comprehensive water quality dataset I found no evidence to suggest that isotope ratios are impacted by long-term water quality parameters. Chapter 3 discusses an applied use of these methods to
assess sewage N pollution to reefs along the Mexican Yucatan peninsula. I show that in developed areas, N isotope ratios are among the highest reported for corals, and these values are correlated with the presence of enteric bacteria. Finally, chapter 4 utilizes a museum collection of gorgonians to reveal long-term trends in C and N isotope ratios across the Wider Caribbean since the mid-1800s. Changes in these values over time suggest that the $^{13}$C Suess effect, and the explosive use of agricultural fertilizers since the 1960s are detectable in these octocorals.
David M. Baker was born on May 29, 1979 in Gettysburg, Pennsylvania. Raised in Maryland, Dave spent his child surrounded by fields and forests, though he found more interesting things to explore in the ephemeral pond in his neighbor’s backyard. A fascination with water took hold in him, and he embarked on the now clichéd timeline of Sunday afternoon runs of Jacques Cousteau as a youth to marine scientist in adulthood.

In 1997, Dave matriculated to St. Mary’s College of Maryland; a fitting place for it’s intimate association with water: the St. Mary’s river and Chesapeake Bay. From the beginning he focused his studies on the marine sciences, and found a natural mentor in his academic advisor, Walter Hatch. Walter exposed Dave to tropical coral reefs, first in Belize, and then in the lab. It was in the lab where Dave’s thumb turned blue from developing the skills needed for growing corals for science and teaching.

Teaching and water led Dave to spend his undergraduate summers with the Living Classrooms Foundation, which paid him (mostly in food) to sail around the Chesapeake Bay and Atlantic, chasing whales and invertebrates with younger minds in pursuit of “learning by doing”. These experiences focused Dave as a teacher, which awkwardly led him to living with his parents while substitute teaching at his old middle school on the subject of sexual education. Dave’s motivations for graduate study should now be clear to the reader.

In desperation for advice, Dave was guided to Kiho Kim at American University, who
graciously offered the poor (and embarrassed) substitute teacher refuge from the perils of the real world. At American, Dave was introduced to a curious group of soft corals, the gorgonians. Over the following 2.5 years Dave would hone his expertise of these corals, and how they can be recorders for environmental change. Kiho educated Dave on how to navigate academia, conduct scientific research, publish findings, and advised him to apply for Ph.D. programs and not waste time with aquariums. From this advice, Dave only listened to the former. However, Kiho didn’t hold this against him, and they remain good friends and colleagues.

Dave finished his Master’s with the firm conclusion that grad school is good. Around this time, Kiho introduced Dave to his former post-doc advisor, Drew Harvell, and her lab group at Cornell University. These folks were investigating the dynamics of diseases afflicting gorgonian corals so Dave was well prepared to join them for his Ph.D. While Drew imparted on Dave her knowledge of all things lacking backbones, Jed Sparks rounded out Dave’s understanding the world of stable isotopes in which we live. Together with Bob Howarth, Dave had a dream-team committee to guide his Ph.D. studies.

After an amazing 8 years since walking away from teaching sex ed, Dave is now ready to re-enter the real world. He begins a post-doctoral position with Marilyn Fogel at the Carnegie Institution’s Geophysical Lab as soon as this dissertation is accepted.
To my parents, Mike and Linda
For letting me choose my own way.
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CHAPTER 1
GORGONIAN CORALS AS RECORDERS OF ENVIRONMENTAL CHANGE:
STABLE ISOTOPE ANALYSIS FOR MONITORING ANTHROPOGENIC POLLUTION

Abstract

Stable isotope analysis of gorgonian octocorals has been highlighted as a cost-effective method for assessing coastal ocean health, particularly for perturbations of carbon (C) and nitrogen (N) cycling in human-impacted regions. Gorgonians preserve information on the predominant sources of C and N assimilated over time as reflected in the ratio of the heavy to light isotope (i.e. δ\(^{13}\)C and δ\(^{15}\)N) of their tissues and skeleton. Still, few studies have investigated the dynamics of uptake and isotopic fractionation mediated by environmental conditions. Here we describe several considerations for the use of gorgonians in isotope studies. We show that common treatments used to remove carbonates, organics, and lipids have effects on δ\(^{13}\)C, but not δ\(^{15}\)N illustrating the simplicity of obtaining simultaneous determination. We test the hypothesis that all gorgonians from a common habitat have similar isotope values and show that there are significant differences between common genera comprising three morphologies; sea rods (Eunicea and Plexaura) have a relatively higher trophic position than sea fans (Gorgonia) and sea plumes (Pseudopterogorgia). Additionally, we rejected the null hypothesis that isotope fractionation is constant with depth. We show that δ\(^{15}\)N decreased along a depth transect in situ, and in an experimental system we demonstrate that light, not temperature, is driving isotope fractionation along depth gradients. However, we predicted that the difference expected across a 30 m depth gradient is less than 0.5 ‰ and therefore not likely to overwhelm signals of
anthropogenic N from highly polluted areas. We reject the hypothesis of uniformity in stable isotope ratios within a colony, since edge samples are lower than samples from the colony center. We can also reject the hypothesis that this pattern arises from age-related changes because small and large colonies from the same environment have the same isotopic values, recorded from changing sources within the environment. Finally, we reject the hypothesis that isotope ratios are static through time because $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ of individually tagged colonies of the common sea fan *Gorgonia ventalina* varied significantly over a three year period on two reefs in the Florida Keys. The variability of individuals through time suggests that these populations are sensitive to changes in the predominant N sources in the region. Significant differences year-to-year coupled with low overall variability in isotope ratios reinforces the idea that gorgonian corals are a superior long-term recorder of environmental quality.

**Introduction**

Human alteration of biogeochemical cycles has increased dramatically since industrialization. Perturbations in the natural flow of elements from source to sink can have negative impacts on the global environment. Beginning with “the Green Revolution” in the 1940’s, widespread production and application of agricultural fertilizers increased global food production. Today, the synthesis of agricultural fertilizers alone exceeds the natural rate of terrestrial N fixation on Earth (Vitousek *et al.*, 1997). At the same time, coastal populations have exploded. In the U.S., 53% of the population lives in coastal areas; a situation that yields increased sewage inputs and atmospheric N deposition to local catchments (Creel, 2007, Galloway *et al.*, 2004, Lapointe *et al.*, 1990). The result is an unprecedented amount of reactive N leaching into watersheds, and eventually to the coastal oceans (Howarth *et al.*, 2000,
An overabundance of sewage and fertilizer-derived N is a critical threat to coastal aquatic ecosystems (Howarth et al., 2000). Much like its intended effect on crops, N boosts productivity in phytoplankton and algae. This leads to precipitous drops in dissolved oxygen, promotes harmful algae blooms, reduces water clarity and quality, and alters the composition and diversity of benthic habitats (Lotze et al., 2006). Nowhere is the latter more evident than on coral reefs (Risk, 2009).

Reef-building corals thrive in warm, low-nutrient waters; conditions under which a mutualistic symbiosis with dinoflagellates evolved. These algae provide the coral host with energy in the form of fixed carbon via photosynthesis, while the coral provides metabolic wastes that, in turn, maintains algal growth. However, with an overabundance of N the mutualism is disrupted, leading to a reduction of coral growth and fecundity (Tomasik & Sander, 1985, Tomasik & Sander, 1987). Furthermore, N changes the community structure of coral reefs. Normally suppressed by the paucity of N in marine waters, macroalgae are quick to smother corals following N enrichment (Lapointe et al., 2005a, Lapointe et al., 2005b). Similarly, competition between corals and heterotrophic fouling organisms such as sponges and mollusks increases in eutrophic conditions (Ward-Paige et al., 2005b). Recent evidence suggests that N exacerbates several coral diseases, a leading cause of coral loss in the Caribbean (Baker et al., 2007, Bruno et al., 2003, Voss & Richardson, 2006). In recent years, the transition of coral reefs to algal-dominated barrens is a common observation worldwide (Halpern et al., 2008, Pandolfi et al., 2003, Somerfield et al., 2008) generating interest in ameliorating pollution at local scales.
The extension of stable isotope methods to coral reef organisms is improving our understanding of pollution sources, especially in areas of significant human development. Specifically, the isotopic ratio of $^{15}$N to $^{14}$N is regarded as an effective, direct indicator for sources of human N pollution. Generally, enriched isotope values (having relatively more $^{15}$N) arise from the accumulation and degradation of human and animal wastes. For example, nitrate in sewage-contaminated groundwater can have $\delta^{15}$N values much greater than 10 ‰ (Katz & Griffin 2008). High $\delta^{15}$N groundwater can impact adjacent ecosystems, and these inputs can be recorded in the isotope ratio of accreting organisms. Ward-Paige et al (2005) used skeletal bands of the gorgonian coral *Eunicea flexuosa* to reconstruct a 25-year N record for reefs in the Florida Keys, revealing a significant increase in $\delta^{15}$N over time due to sewage N pollution. Similarly, agricultural fertilizers are easily distinguished from natural N sources, and these sources tend to decrease $\delta^{15}$N in impacted ecosystems, as the isotope ratios are low, averaging -1 to 0 ‰. Marion et al (2006) reported a significant (~7.0 ‰) depletion in skeletal bands from cores of the massive coral *Porites sp.* growing adjacent to agricultural activities in Indonesia relative to offshore, which were highly correlated with increased agricultural fertilizer use. Furthermore, isotopic analyses of deep-sea gorgonian skeletons have been shown to track surface water processes. Sherwood et al. (2005) demonstrated that the isotopic and elemental profiles of skeletal proteins from modern and fossil specimens of *Primnoa spp.* were similar, and suggested that isotopic profiles of ancient corals can be used to reconstruct past environmental conditions over millennial timescales.

Species-specific physiology and response to environmental drivers may impact isotopic signatures in unknown ways. For example, $^{18}$O/$^{17}$O ratios of scleractinian coral skeletons have been used as a proxy for past sea surface temperatures. A recent
study of stony corals in Hawaii found that $d^{18}$O values did not accurately reflect sea surface temperatures when corals are bleached due to kinetic fractionations associated with calcification (Rodriquez & Grottoli 2007). It’s generally accepted that $\delta^{15}$N is a good indicator of sewage and fertilizer N (Heaton, 1986, Mayer et al., 2002), and many studies have supported this idea by documenting gradients of increasing (sewage) or decreasing (fertilizer) $\delta^{15}$N values in reef biota (Heikoop et al., 2000, Lapointe et al., 2004, Marion et al., 2005, Risk & Erdmann, 2000, Risk & Heikoop, 1997). However, Swart et al. (2005) found no link between tissue $\delta^{15}$N of Montastrea faveolata and distance from shore in the Florida Keys, and raised the point that $\delta^{15}$N as a tracer for pollution may be problematic. However, Risk et al. (2009) have questioned the results of Swart et al. (2005), citing a lack of attention to potential confounding influences of light levels between turbid nearshore sites and clearer offshore reefs. Furthermore, aside from the organic tissues of hard corals, the underlying inorganic skeleton is comprised of a very small organic fraction, which can be further confounded by bioerosion and endolithic organisms making isotopic assessments of N within hard corals difficult.

Recent reviews by Risk (2009) and Risk et al. (2009) have highlighted gorgonian octocorals as an ideal target for stable isotope studies for assessing sewage stress on reefs. While stony corals are useful in that individual colonies can hold centuries-long records for environmental change within their inorganic skeleton (e.g. temperature, salinity, ocean circulation), gorgonian corals have ample organic material for analysis. The organic components of stony corals are the thin veneer of living tissue atop the skeleton and the matrix “scaffolding” used for skeletogenesis, which comprises less than 0.015% of the skeletal mass (Marion et al., 2005). Gorgonians secrete a proteinaceous (gorgonin) skeleton that is rich in C and N and is resistant to diagenesis
(Goldberg, 1974, Goldberg, 1978, Sherwood et al., 2005). The individual coral polyps extend into the environment from apertures; openings in the common tissues that surround the skeleton (also known as the “axis”). The polyps are connected via a network of vessels, allowing for the exchange of nutrients and wastes throughout the colony. Previous work has shown no difference in $\delta^{15}N$ between the tissue and skeletal components (Baker et al., 2007, Heikoop et al., 2002), though there have been no studies to determine how nutrients flow between the environment and the colony, and how these nutrients are distributed within the colony between newly growing and old tissues.

$\delta^{13}C$ determinations are not as straightforward for gorgonians, which possess several pools of carbon that are heavily fractionated or non-dietary in origin, and therefore contribute noise to dietary or anthropogenic signals. For instance, some gorgonians, like the common sea fan *Gorgonia ventalina*, are dioecious. Females develop eggs throughout the year until summer spawning, while others fertilize internally and brood the larvae on the coral surface as in the case of *Briareum asbestinum* (Harvell et al., 1996). As coral eggs and larvae are lipid rich (Alamaru et al., 2009b), females with developed ova or colonies brooding larvae may have a higher proportion of lipids than conspecifics. Lipids have unique isotopic signatures due to fractionations during lipid synthesis (DeNiro & Epstein, 1977). Thus, it is possible that individual variations in $\delta^{13}C$ are due to differences in sex or reproductive stage. Additionally, most gorgonians have unique, calcitic structures within the coenenchyme surrounding the skeletal axis (Harvell et al., 1988). The C in these sclerites is mostly derived from seawater bicarbonate, and therefore differs isotopically from dietary sources (Lucas & Knapp, 1997). Furthermore, sclerites can vary between individuals, at times increasing due to stress from predation or intense wave action (West, 1998).
Nutritionally, symbiotic corals are “polytrophic”, acquiring energy and nutrients from autotrophy through their symbiotic algae as well as heterotrophy via predation on a wide range of plankton (Porter, 1976). As $\delta^{15}$N is dependent on an organism’s trophic status (Deniro & Epstein, 1981, Minagawa & Wada, 1984), species that tend towards heterotrophy will have higher $\delta^{15}$N relative to more autotrophic corals. Similarly, $\delta^{13}$C increases relative to trophic position, though only by an average $\sim$1.0 ‰ per trophic level. Furthermore, plankton have more depleted $\delta^{13}$C relative to bicarbonate, therefore heterotrophic corals will have more depleted $\delta^{13}$C than obligate autotrophs. As a whole, gorgonians display a wide variety of growth forms, which are hypothesized adaptations for prey capture (Leversee, 1976). Corals that are more heterotrophic, (facultative autotrophs) can acquire nutrients from multiple sources and trophic levels, which could potentially confound interpretation of isotope ratios. For example, Alamaru et al. (2009) showed that the relative heterotroph Favia favus, did not mirror trends in lipid $\delta^{13}$C along a depth gradient exhibited by the more autotrophic Stylophora pistillata (Alamaru et al., 2009a). Conversely, obligate autotrophs may accurately record sources of inorganic nutrients with high fidelity (Baker et al. in preparation).

Autotrophy is also an important factor as the metabolism and growth of symbiotic corals is highly influenced by light. High metabolic rates are associated with reduced isotopic fractionation and vice versa (Heikoop et al., 1998, Muscatine et al., 1989). Therefore, with all else being equal, corals growing in a high light environment (shallow waters) will be enriched relative to conspecifics at low light (deeper waters). However, light-mediated isotope effects have yet to be documented in gorgonian corals (Risk, 2009).
This study investigates the utility of gorgonian isotope ratios as a record for environmental nutrient sources with a primary focus on $\delta^{15}$N. However, as simultaneous determinations of $\delta^{13}$C are common during isotope ratio mass spectrometry, we include these data where appropriate. We began by testing the null hypothesis that tissue and axis components of gorgonians are not affected by common pre-analysis treatments. Using this information, we normalized museum-collected specimens of 4 genera within the Suborder Holaxonia (Family Gorgoniidae & Plexauridae), to determine the relative trophic position of common groups. We then identify the genera that appear to be lowest on the marine food web as indicated by their isotope ratios, and therefore most likely to record inputs of inorganic N. Using these candidate species, we test the hypothesis that isotope ratios vary with depth, and experimentally evaluate which aspect of depth (light or temperature) influences isotope fractionations. We test various pre-analytical treatment effects including acid, bleach, and dichloromethane, for the removal of organics, carbonates, and lipids, respectively. Lastly, we test the hypothesis that isotope ratios are stable with time and space using a wild population of tagged sea fans in the FKNMS, which were sampled annually for three years.

Methods

Treatment Effects

We used 2 separate experiments to test for the impact of common pre-treatment methods for stable isotope analysis. First, we analyzed 24 sea fan samples from the Florida Keys, collected in the summer of 2000 (Baker et al., 2007, Dube et al., 2002).
The tissue and axis components were separated, and these components were divided for treatment in either 6N HCl (treatment), or deionized water (control) for 24 hours. Following this treatment the samples were rinsed repeatedly with deionized water and oven-dried for 24 hours at 60 C.

In a second experiment, we sampled three whole (~30 cm) colonies of *G. ventalina*. A 2 x 2 cm section of the colony edge, and a 2 x 2 cm section of the lower interior adjacent to the primary axis were sampled, and separated into tissue and axis components. These sites on the colony were chosen to test the hypothesis that tissue and axis differ throughout the colony. These components were then sub-sampled for combinations of treatments with a 50% commercial bleach solution, and lipid extraction with dichloromethane.

**Diversity Study**

Variation in δ¹⁵N and δ¹³C between genera was determined from a museum collection of gorgonian corals collected between 1861-2005 across their natural range within the Wider Caribbean. Geographic bias does not confound our analyses as many of the genera were collected from the same locations, and the large sample sizes should minimize any impact of geography. Dry museum specimens were obtained from the Smithsonian National Museum of Natural History Invertebrate Zoology Collection (Washington, DC), the Harvard University Museum of Comparative Zoology (Cambridge, MA) and the Yale Peabody Museum (New Haven, CT). We targeted the tips of the most distal branches or plane of the coral, which represent an approximate integration of the C and N sources assimilated during the most recent growth (Cary, 1914). In *Gorgonia*, we sampled a continuous patch, approximately 2 x 1 cm from the
colony edge. For *Pseudopterogorgia*, *Plexaura*, and *Eunicea*, we sampled approximately two branches, 3 – 5 cm in length from the distal end of each specimen. Samples were removed using scissors. In preparation for $\delta^{13}C$ determination, samples were acidified to remove carbonates from sclerites via exposure to a concentrated HCl atmosphere for a minimum of 48 hours or until bubbling was no longer observed in a replicate sample when concentrated HCl was added drop-wise (Hedges & Stern, 1984, Jaschinski et al., 2008). After oven drying for 24 hr at 60 C to remove excess acid, soxhlet extractions were performed using 83:17 (v:v) chloroform:methanol to remove lipids. Samples were again oven-dried at 60 C for 24 hours.

*Collections for depth analysis*

The effects of depth on $\delta^{15}N$ were examined using *Pseudopterogorgia americana* collected along a depth gradient at Molasses Reef, Florida Keys, USA, in August of 2006. *P. americana* was selected because of its local abundance across a relatively large depth range. A sample was collected every 0.3 m from 4.3 to 13.4 m depth. Samples were air-dried and isotope analysis was conducted as described below. Effects of depth on $\delta^{15}N$ were determined using a regression analysis.

*Laboratory test for the effects of light and temperature on $\delta^{15}N$*

As both temperature and light decrease with depth, we manipulated these parameters in a 6-month laboratory study to assess their relative contribution to $\delta^{15}N$ fractionation. Three colonies of *P. americana* collected from the Lower Florida Keys were clonally fragmented into ~4 cm length branches ($n = 54$) which were glued to a concrete base and allowed to recover in a recirculating seawater system at Cornell
University for 2 weeks. Subsequently, one fragment of each clone was placed in a 1.5 L plastic container haphazardly assigned to one of six light treatments (61, 69, 74, 83, 95, and 100% surface PAR measured by a $4\pi$ quantum sensor) achieved by using layers of shade cloth under a combination of broad-spectrum and actinic very high output (VHO) compact fluorescent lighting (Tek-Light, 6 x 54 W; 14:10 light:dark). These six light shaded containers were generated in triplicate, and each set of six was placed into 200 L tanks, partially filled with deionized water as a water bath. These baths were used to regulate the temperature for each set of containers to 25, 28, and 30 degrees C, respectively, using 200W submersible heaters. Each 1.5 L container was equipped with an airstone to provide circulation around the corals, and the flow of air was carefully regulated with a manifold to ensure even rates of circulation within each container. Every 3 days, a complete water change was performed on each container, and the containers were rotated within the water bath to minimize tank effects from variations in light and/or circulation. Corals were fed ~ 5 mL of a suspension of small particle food (Roti-Rich™, FAFUSA; $\delta^{15}N \approx 0.0 \%$) and ~40 mm oyster eggs (DT’s Plankton Farm, Sycamore, IL, USA; $\delta^{15}N \approx 7.5 \%$) as a source of N. After 6 months, the corals were removed from the aquaria, oven-dried and the tissue was separated from the skeletal components. The tissue was then prepared for isotope analyses as described below.

**Stable isotope analyses**

All samples were homogenized by grinding using a mortar and pestle, or in a Certiprep SPEX cryogrinder using liquid N. The resulting powder, consisting of the skeletal axis, and/or coenenchyme, polyps, and zooxanthellae was weighed into 4 x 6 mm tin capsules, which were combusted in a Carlo-Erba elemental analyzer and
analyzed by a Finnegan-MAT Delta Plus isotope ratio mass spectrometer. Reported \( \delta^{15}\text{N} \) and \( \delta^{13}\text{C} \) values are relative to atmospheric N\(_2\) and Vienna Pee Dee Belemnite (VPDB), respectively. Precision for \( \delta^{15}\text{N} \) and \( \delta^{13}\text{C} \) was quantified by two in-house standards [bcbg (0.08 and 0.06 ‰, respectively) and methionine (0.09 and 0.1 ‰, respectively)] calibrated against IAEA international standards. Additionally, we used an in-house homogenized sea fan standard, which had a precision for \( \delta^{15}\text{N} \) and \( \delta^{13}\text{C} \) of 0.05 and 0.25 ‰, respectively.

Annual variation in isotope values in two populations of sea fans

To determine annual and population level variation of \( \delta^{15}\text{N} \) and \( \delta^{13}\text{C} \), 20 colonies of *G. ventalina* were tagged at Carysfort Reef and Pickles Reef in the Florida Keys in July 2005. A 2 cm\(^2\) fragment was removed from the colony edge using scissors. The samples were held on ice, and air-dried. Colonies were re-sampled in 2006, and 2007.

Statistical Analyses

Prior to analysis, all data were screened for normality and homoscedasticity using Shapiro-Wilk and Levene’s tests, respectively, and transformed as necessary to meet the assumptions for parametric statistical tests. Unless otherwise noted, the data met these assumptions without requiring transformations. First, pair-wise t-tests were used to compare the means of sea fan components subjected to various treatments, relative to controls as well as outer vs. inner portions of the colony. Second, we used analysis of variance (ANOVA) to test the hypothesis that \( \delta^{15}\text{N} \) and \( \delta^{13}\text{C} \) differ between genera, and *post-hoc* pair-wise t-tests to assess significant differences between genera. Third, we used linear regression to assess the relationship between colony size of individual
colonies of *G. ventalina* and their corresponding isotopic and elemental ratios. Fourth, this analysis was again used to test the hypothesis that $\delta^{15}\text{N}$ varies with depth from field collected *P. americana*. Fifth, a linear model was used to assess the effect of clone, temperature, light, and the interaction of temperature and light on $\delta^{15}\text{N}$ values from the laboratory cultured *P. americana*. A random-effects model with clone as the random factor was then used to further describe the relationship between $\delta^{15}\text{N}$ and light. Finally, ANOVA was used to test the hypothesis that isotope ratios vary year-to-year in two populations of *G. ventalina* at Carysfort and Pickles reef. Post-hoc t-tests were used to identify significant difference within reefs by year. Pair-wise t-tests were then used to test the hypothesis that isotope ratios differed between reefs within a given year.

From these population-level data, we used power analysis to determine the minimum sample sizes of *G. ventalina* needed to detect significant differences between sites for various hypothetical differences to detect. All statistical analyses were conducted using JMP v.7.0 (SAS, Cary, NC).

**Results**

**Treatment Effects**

Treating both the axis and tissue components separated from *G. ventalina* with dilute HCl produced slight depletions in both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. However, neither C nor N isotope ratios were significantly different from controls in axis samples (Table 1.1). The slight depletion in $\delta^{15}\text{N}$ observed in the acid washed tissue samples was also not statistically different from controls, however, acid washing significantly depleted the
Table 1.1. Summary of the effects of pre-treatments on the isotope ratios of tissue and axis components of the common sea fan *Gorgonia ventalina*. *P*-values in **bold** signify significant differences between the acid treatment and control.

<table>
<thead>
<tr>
<th>Test</th>
<th>Component</th>
<th>Value</th>
<th>df</th>
<th>Control (mean ‰ ± SE)</th>
<th>Treatment (mean ‰ ± SE)</th>
<th>Δ (‰)</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid</td>
<td>Tissue</td>
<td>δ¹⁵N</td>
<td>44</td>
<td>5.7 ± 0.3</td>
<td>5.5 ± 0.3</td>
<td>-0.2</td>
<td>-0.39</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>δ¹³C</td>
<td>46</td>
<td>-6.3 ± 0.5</td>
<td>-8.6 ± 0.5</td>
<td>-2.3</td>
<td>-2.99</td>
<td><strong>0.004</strong></td>
</tr>
<tr>
<td></td>
<td>Axis</td>
<td>δ¹⁵N</td>
<td>45</td>
<td>6.0 ± 0.2</td>
<td>5.7 ± 0.2</td>
<td>-0.3</td>
<td>-1.39</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>δ¹³C</td>
<td>45</td>
<td>-13.8 ± 0.4</td>
<td>-15.1 ± 0.5</td>
<td>-1.3</td>
<td>-1.88</td>
<td>0.07</td>
</tr>
<tr>
<td>Bleach</td>
<td>Tissue</td>
<td>δ¹⁵N</td>
<td>10</td>
<td>3.0 ± 0.3</td>
<td>2.9 ± 0.3</td>
<td>-0.1</td>
<td>0.21</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>δ¹³C</td>
<td>10</td>
<td>-11.2 ± 0.3</td>
<td>-11.3 ± 0.3</td>
<td>-0.1</td>
<td>0.14</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>Axis</td>
<td>δ¹⁵N</td>
<td>10</td>
<td>3.1 ± 0.1</td>
<td>3.3 ± 0.1</td>
<td>-0.2</td>
<td>-1.48</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>δ¹³C</td>
<td>10</td>
<td>-16.0 ± 0.3</td>
<td>-16.9 ± 0.3</td>
<td>-0.9</td>
<td>-2.53</td>
<td><strong>0.03</strong></td>
</tr>
<tr>
<td>DCM</td>
<td>Tissue</td>
<td>δ¹⁵N</td>
<td>10</td>
<td>3.0 ± 0.4</td>
<td>2.6 ± 0.4</td>
<td>-0.4</td>
<td>-0.67</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>δ¹³C</td>
<td>10</td>
<td>-11.2 ± 0.3</td>
<td>-10.5 ± 0.3</td>
<td>-0.7</td>
<td>-1.83</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>Axis</td>
<td>δ¹⁵N</td>
<td>10</td>
<td>3.1 ± 0.1</td>
<td>3.2 ± 0.1</td>
<td>0.1</td>
<td>0.56</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>δ¹³C</td>
<td>10</td>
<td>-16.0 ± 0.2</td>
<td>-16.1 ± 0.2</td>
<td>0.1</td>
<td>-0.32</td>
<td>0.75</td>
</tr>
</tbody>
</table>

δ¹³C of tissue by 2.3 ‰ indicating removal of carbonates (Table 1.1). Bleach treatment caused a significant 1.7 ‰ depletion in δ¹³C of the axis (t-test, *n* = 6, *t* = 3.75, *p* = 0.02), however, a bleach effect was not observed when samples were also lipid extracted with DCM, suggesting that either treatment successfully removed lipids left over in remnant tissue on the axis.

**Gorgonian Diversity**

There were significant differences between 4 genera of gorgonians sampled from a museum collection for both δ¹⁵N (ANOVA; *F*₃,₁₇²* =* 4.16, *p* = 0.007) and δ¹³C (*F*₃,₁₇¹* =* 41.98, *p* < 0.0001; Table 1.2, Fig. 1.1). *Eunicea* were the most enriched in both δ¹⁵N and δ¹³C, and were not significantly different from *Plexaura* with respect to δ¹⁵N. Sea
Table 1.2. Summary of stable isotope values by genus from a museum collection of gorgonian corals. Values not sharing the same letter are significantly different (*post-hoc* Student’s t-tests, $\alpha = 0.05$)

<table>
<thead>
<tr>
<th>Genus</th>
<th>n</th>
<th>Mean $\delta^{15}$N (‰ ± SE)</th>
<th>Mean $\delta^{13}$C (‰ ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Eunicea</em></td>
<td>60</td>
<td>4.4 ± 0.2$^a$</td>
<td>-7.7 ± 0.5$^a$</td>
</tr>
<tr>
<td><em>Plexaura</em></td>
<td>16</td>
<td>4.2 ± 0.3$^{a,b}$</td>
<td>-9.9 ± 0.9$^b$</td>
</tr>
<tr>
<td><em>Pseudopterogorgia</em></td>
<td>68</td>
<td>3.9 ± 0.2$^{b,c}$</td>
<td>-14.8 ± 0.4$^c$</td>
</tr>
<tr>
<td><em>Gorgonia</em></td>
<td>27</td>
<td>3.4 ± 0.3$^c$</td>
<td>-11.7 ± 0.7$^b$</td>
</tr>
</tbody>
</table>

Figure 1.1. Mean $\delta^{15}$N vs. mean $\delta^{13}$C of 4 genera of gorgonian corals. Bars represent standard error.

fans (*Gorgonia*) were the most depleted with respect to $\delta^{15}$N, while sea plumes (*Pseudopterogorgia*) were most depleted in $\delta^{13}$C. However, the average $\delta^{15}$N value of *Gorgonia* was not significantly different from *Pseudopterogorgia*, nor was mean $\delta^{13}$C different from that of *Plexaura* (Table 1.2).
Intra-colony variability and size/age effects

\[ \delta^{15}N \] of tissue samples from the perimeter of 3 sea fan colonies were significantly depleted, by 1.5 \%, relative to tissue from the colony base (two-tailed t-test, \( n = 6, t = 4.97, p = 0.008 \)). C:N ratios were higher, by 0.37, in the younger skeletal axis at the perimeter of the colony, relative to the older skeleton at the base (t-test, \( n = 6, t = -3.7, p = 0.02 \)). There was no relationship between colony height and \( \delta^{15}N, \delta^{13}C, \) or C:N in \textit{G. ventalina} sampled from the Florida Keys (Table 1.3).

Table 1.3. Results of correlation analyses between colony height (cm) and isotope and elemental ratios of \textit{G. ventalina}.

<table>
<thead>
<tr>
<th>Value</th>
<th>( n )</th>
<th>( R^2 )</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \delta^{15}N )</td>
<td>31</td>
<td>0.06</td>
<td>0.18</td>
</tr>
<tr>
<td>( \delta^{13}C )</td>
<td>32</td>
<td>0.10</td>
<td>0.09</td>
</tr>
<tr>
<td>C:N</td>
<td>32</td>
<td>0.03</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Depth Sampling Effects

\( \delta^{15}N \) values of the sea plume, \textit{Pseudopterogorgia americana} collected along an 8.2 m depth gradient ranged from 3.2 to 4.0 \%, becoming progressively depleted with increasing depth at a rate of 0.061 \%\textperthousand \textperthousand m\(^{-1} \) (Fig. 1.2).

The \( \delta^{15}N \) values of \textit{P. americana} cultured during a 6 month laboratory experiment were not normally distributed. To achieve normality, \( \delta^{15}N \) values were Box-Cox transformed. The resulting transformed values were normal (Shapiro-Wilk test, \( p = 0.06 \)) and homoscedastic (Levene’s test, \( p = 0.89 \)). ANOVA revealed a significant effect of light and clone, but not temperature on \( \delta^{15}N \) (Table 1.4). A random-effects model with clone as the random factor and light as the model effect accounted for 50\%
of the variation in $\delta^{15}N$, with a significant effect of light ($p = 0.001$). The resulting linear model defining the relationship between $\delta^{15}N$ and depth predicted a 0.014 ‰ decline in $\delta^{15}N$ per meter depth (Fig. 1.3), with samples exposed to the highest light intensity being most similar to the source. Over the simulated 30 m depth range we would expect a 0.42 ‰ deviation in $\delta^{15}N$ due to light isotope effects alone.
Figure 1.3. $\delta^{15}$N of the sea plume *Pseudopterogorgia americana* as a function of depth, estimated from % surface PAR, after a six-month incubation at six light intensities and three temperature treatments. The $\delta^{15}$N of the source N is shown as a dotted line. Bars represent standard error. Line represents a significant linear relationship.

*Annual variability and power analysis*

Tagged colonies of *G. ventalina* at Carysfort and Pickles reef in the Florida Keys, sampled in 2005, 2006, and 2007 showed significant differences in $\delta^{15}$N by year (Carysfort, $F_{2,50} = 22.73$, $p < 0.0001$; Pickles, $F_{2,36} = 10.05$, $p < 0.0004$; Fig. 1.4a), but only at Carysfort had a significant effect of year on $\delta^{13}$C (Carysfort, $F_{2,49} = 5.40$, $p = 0.008$; Pickles, $F_{2,36} = 1.19$, $p = 0.32$; Fig. 1.4b). However, $\delta^{15}$N and $\delta^{13}$C values were similar between the two sites in all years, with the exception of 2006, when $\delta^{13}$C was ~0.6 ‰ higher at Pickles reef. There was a ~0.2 ‰ increase in $\delta^{15}$N from 2005 to
Figure 1.4. δ¹⁵N (A) and δ¹³C (B) of 20 tagged colonies of *Gorgonia ventalina* sampled within the Florida Keys National Marine Sanctuary from Caryfort (red) and Pickles (blue) reef from 2005 to 2007. Letters represent statistically similar annual means within a site, while asterisks signify significant differences between sites within a year.
2006, followed by a decline between 2006 and 2007 of ~0.3 ‰ (Fig. 1.4a). These small fluctuations are close to the analytical precision of the IRMS, though all samples were run simultaneously. The pattern was opposite for $\delta^{13}$C which was slightly lower in 2006 by ~0.4 ‰, though this difference was not significant for Carysfort reef (Fig. 1.4b). There was no difference in either $\delta^{15}$N or $\delta^{13}$C between these sites in any given year, with the exception of $\delta^{13}$C in 2006, which was significantly lower in Pickles relative to Carysfort.

A critical first question in designing an isotope monitoring experiment is to select a target organism for sampling. Organisms with naturally high variability will have less power to detect small differences in isotope ratios between locations or over time, unless very large sample sizes are used which adds considerable costs. We used a power analysis to answer the question, how many samples would we need to detect a significant difference between mean $\delta^{15}$N at Carysfort vs. Pickles? Our results suggest that detecting a difference between these reefs less than 0.5 ‰ should be possible with 5 samples per site (Fig. 1.5).

**Discussion**

This study contributes to our current knowledge of gorgonian corals, and supports previous claims as to their utility for isotopic assessments of anthropogenic impacts to coral reef ecosystems, though the considerations herein can be applied towards the development of any biological isotopic integrator (Baker *et al.*, 2007, Risk, 2009, Risk & Heikoop, 1997, Risk *et al.*, 2009, Ward-Paige *et al.*, 2005a). The results we present are vital to informing future research and coral reef management. When careful considerations for the confounding effects of comparing different sample preparations,
Figure 1.5. Results of power analysis of various differences to detect scenarios, based on the standard deviation of $\delta^{15}$N from sea fans collected from Carysfort reef between 2005-2007.

taxa, locations, and sampling times, there is a high likelihood of cost-effective detection of human impacts on the coastal ocean, before substantial habitat degradation occurs (McCook, 1999).

*Treatment effects on gorgonians*

This is the first study to quantify the effects of treatments for removing compounds that are commonly perceived as confounding in stable isotope analyses of gorgonian corals. In gorgonians, sclerites provide structural support and anti-predator defenses, and can vary naturally in the environment. They are also composed of aragonitic calcium carbonate, which is derived from seawater, and thus contain a source of
carbon that is not dietary in origin. To obtain a record of dietary C, sclerites should be removed prior to $\delta^{13}C$ determination via acidification (Hedges & Stern, 1984, Jaschinski et al., 2008). Acids dissociate the ionic bond and generate CO$_2$ from the carbonate molecule, which is lost to the atmosphere. Acid depleted all isotope values, both tissue and skeleton, but only affected $\delta^{13}C$ in the tissue significantly via sclerite removal. This also suggests that the sclerites do not contain a unique or substantial source of N because $\delta^{15}N$ was not affected. The slight but non-significant effect of acid washing on $\delta^{13}C$ values of the axis was likely due to incomplete separation of tissue containing sclerites. Similarly, bleach caused a slight depletion in the $\delta^{13}C$ of the axis, which again was likely due to removal of residual tissues adhering to the skeleton. Though in this case, as bleach does not dissolve sclerites, it probably loosened them by oxidizing the mucopolysaccharides and glycoproteins of the coenenchyme that ‘glue’ them together against the axis, causing them to fall away from the skeleton.

Lipid extraction had a negligible effect on both isotopes in axis and tissue. There was a slight depletion in tissue treated with DCM, suggesting that lipids may need to be normalized in studies concerned with comparing $\delta^{13}C$ between taxa, sites, or times with high precision. Ultimately, there was very little effect observed by any of the applied treatments on $\delta^{15}N$, confirming yet again the robust stability of N compounds in gorgonians (Sherwood et al., 2005). As $\delta^{13}C$ and $\delta^{15}N$ determinations are commonly obtained from the same sample, individual gorgonian samples can be prepared with dual analysis in mind, without the need for separate runs for C and N.
Implications for diversity and stable isotope analyses

There is conjecture that in corals, increasing polyp size is indicative of higher trophic position (Porter, 1976). In shallow, symbiotic gorgonians, studies have examined the relative contribution of auto- vs. heterotrophy to coral growth and reproduction, by analyzing gut contents and particle capture experiments (Lasker, 1981, Ribes et al., 1998). Results suggest that zooxanthellate gorgonians have differential abilities in capturing prey and have different polyp extension behaviors, yet heterotrophy does not appear to be a substantial source of nutrition (Ribes et al., 1998). As an alternative to gut and behavior analyses, stable isotope values, particularly for C and N, allow us to make inferences as to the relative trophic position of organisms. $\delta^{15}N$ and $\delta^{13}C$ increase as you move up the food web by 3.4 and 1.0 ‰ per trophic level, respectively (Minagawa & Wada, 1984). We found differences between gorgonian genera, normalized by removing carbonates and lipids prior to analysis. On the surface these differences appear to be due to separation by trophic position. The $\delta^{15}N$ of sea rods suggest that they are positioned higher on the marine food web than sea fans and sea plumes (Fig. 1.1). Interestingly, these patterns, particularly for $\delta^{15}N$, appear to correspond with polyp size in these genera. Eunicea and Plexaura have polyp apertures ~6.0 mm², Pseudopterogorgia apertures are half this size, and those of Gorgonia are less than 1 mm². Porter (1976) speculated that polyp size is a reflection of relatively higher heterotrophy in corals. However, recent work has shown that there is no relationship between polyp size and $\delta^{15}N$ in hard corals (Yamamuro et al., 1995) and an even wider range of gorgonian species (Webster, unpubl.). It’s possible that the scale of our sampling, or species-specific (as opposed to genera specific) bias is affecting our interpretation of these data.
Carbon isotope ratios from the museum-collected specimens revealed the opposite pattern. *Eunicea* and *Plexaura* had the most enriched $\delta^{13}C$ values, while *Pseudopterogorgia* and *Gorgonia* were the most depleted. This pattern suggests that the latter two genera are relatively more heterotrophic with respect to C. Enriched $\delta^{13}C$ values would arise from uptake of bicarbonate, which can be converted to CO$_2$ for photosynthesis. The small difference between the $\delta^{13}C$ reported in these genera and the $\delta^{13}C$ of bicarbonate (~ 7 ‰) signifies efficient uptake and retention of bicarbonate C, with little fractionation. *Pseudopterogorgia* and *Gorgonia* have $\delta^{13}C$ values similar to plankton (-18 to -17 ‰ (Land *et al.*, 1975), and are 2 ‰ and 5 ‰-enriched relative to this potential source. Average trophic enrichments for C average 1 ‰, therefore *Pseudopterogorgia* appears to acquire a major source of C from a plankton diet, while *Gorgonia* may acquire a portion of it’s C from both bicarbonate and dietary sources.

However, if we rely on N isotope values as indication of relative heterotrophy, then sea rods may be more appropriate integrators of diet (e.g. plankton consumption). Plumes and fans on the other hand, are relatively low in $\delta^{15}N$, which could be an indication that these taxa are more autotrophic, assimilating inorganic forms of N, and not relying as much on heterotrophy. Based on our data, sea fans and sea plumes are better candidates for studies concerned with monitoring change in inorganic pools of N (Baker *et al.* in preparation). Indeed, $\delta^{15}N$ of *G. ventalina* closely matched the $\delta^{15}N$ of nitrate, within 0.1 ‰ in a coastal lagoon in Mexico (Baker *et al.* in preparation; (Mutchler *et al.*, 2007). Our conclusions suggest that direct comparison between the results of Baker *et al.* (2007) on *G. ventalina*, and Ward-Paige *et al.* (2005) on *E. flexuosa*, are not possible due to the separation in the relative trophic positions of sea fans and sea rods. Future research should continue to focus on the implications of
diversity on stable isotope ecology of gorgonians, particularly to identify which species are comparable across studies.

*Intra-colony variability and size effects*

Comparisons of tissues and skeleton of both a shallow-water (*G. ventalina*) and deep-water (*Primnoa*) gorgonian species have shown that there is no difference in $\delta^{15}$N (Baker et al., 2007, Heikoop et al., 2002). However, within-colony variation in isotope ratios has not been examined in shallow-water gorgonians, nor has the effect of colony size. We detected significant variability between perimeter and interior samples from just three colonies of *G. ventalina*. $\delta^{15}$N was on average 1.5 ‰ lower in tissue from the edge of the colony relative to the interior. Also, C/N of the axis was slightly higher in the perimeter samples. Given that concentric growth rings of the gorgonian skeleton can record annual variation in $\delta^{15}$N (Ward-Paige et al., 2005a, Williams et al., 2007), and 2) there is no difference between the tissue and axis components at the colony perimeter (Baker et al., 2007, Heikoop et al., 2002), we hypothesize that tissue $\delta^{15}$N does not turnover during growth, thus the environmental conditions recorded in the tissue at the time of growth are recorded much like the skeleton. Further work must be conducted to explain these patterns in more detail, specifically greater sub-sampling within individual colonies along a base – perimeter transect, coupled with analyses of skeletal growth rings would be useful for comparing the records stored within each component. In the meantime, sampling from the distal edges of gorgonians is preferable, as they likely contain information on the sources of C and N acquired during the most recent growth (6-12 months).
We hypothesized that ontogenetic dietary shifts would be detectable in large individuals. Our analysis of tagged *G. ventalina* colonies from the Florida Keys refutes this hypothesis, as there was no relationship between colony height and either isotope or elemental ratios from samples taken at the perimeter (Table 1.3). This lends further support that these colonies, regardless of size/age, are recording environmental conditions. Overall, there was very low variability among these corals (Fig. 1.4), suggesting that size is not a concern for isotope sampling, though we cannot extrapolate further than the size class we sampled (between 30 – 100 cm). However, colonies in this size class are common and conspicuous and therefore an ideal target for stable isotope sampling.

*Light-mediated isotope fractionation in gorgonians*

Heikoop *et al.*, (1998) demonstrated that $\delta^{15}$N values in hard corals from Jamaica and Zanzibar are positively correlated with light exposure as evidenced by an average drop of 0.05 ‰*m$^{-1}$. Over the 29m depth range sampled in their study, light explained a ~1.4‰ difference between the $\delta^{15}$N values of shallow vs. deep corals. Similarly, we observed a rate of decrease in $\delta^{15}$N of 0.061 ‰*m$^{-1}$, which by extrapolation, would amount to a 1.8 ‰ shift in $\delta^{15}$N along a similar depth gradient. Thus, light is a probable driver for isotope fractionations along a depth gradient in gorgonians. In high light environments, photosynthetic rates are elevated which increases the internal demand for N, depletes the internal N pool, and consequently eliminates any isotopic fractionation during the process of assimilation to incorporation in tissues. Conversely, in low light environments photosynthetic rates are reduced, allowing for the build-up of an internal N pool. Selective assimilation of $^{14}$N from this pool lowers the $\delta^{15}$N of the coral relative to the source.
Increasing metabolic rate (photosynthesis) is the proposed mechanism for light mediated isotope fractionation (Heikoop et al., 1998, Muscatine et al., 1989). For invertebrates, temperature should also have a strong impact on metabolism. As temperature also varies with depth, we used a long-term laboratory study to evaluate the relative influence of both light and temperature on δ¹⁵N. Our data show that temperature has no effect on isotope fractionations within the 5 C range we used. Beyond this range we approach the limits of thermal tolerance of these tropical species. Light on the other hand, had a strong affect on δ¹⁵N in our experiment illustrating a pattern consistent with the mechanism proposed by Heikoop et al. (2000). At the highest light intensity fractionation was low between the coral δ¹⁵N and their food source (Δ¹⁵N = 0.1 ‰). Conversely, under low light conditions the corals were offset from their food source by ~0.6 ‰. This offset was likely due to lower photosynthetic rates, resulting in a lower internal demand for N, and thus a larger N pool from which preferential assimilation of ¹⁴N does not limit growth and maintenance of tissues.

The slopes of the best-fit lines from the field collection and lab experiment (Figs. 1.2 & 1.3) differed by 0.05 ‰*m⁻¹. The discrepancy between these slopes could be due to variable sources encountered in the field, whereas N source was controlled in our lab experiment. By extrapolation of the best-fit lines, we can predict the difference in δ¹⁵N expected along a 30 m depth gradient is ~1.4 ‰. The larger slope determined from the field collected samples could be due to source effects across the reef slope. For example, if upwelling is the dominant N source to these corals, then deeper water corals are less likely to be limited by N. Corals in shallower waters, being more N-limited and having a higher N demand due to the high light environment will be
constrained to utilizing the remnant N pool, which will become progressively enriched as $^{14}\text{N}$ is preferentially assimilated along the reef slope. The much lower light effect observed in the lab suggests that light has a significant, though minor influence on $\delta^{15}\text{N}$ and the range we report here from the field collections are influenced by natural source variability. Provided that depth is controlled during isotope sampling in the field, direct comparisons are possible between sites at similar depths to infer relative differences in sources, especially when the differences to detect are larger than 0.5 ‰.

_Monitoring sea fan populations in Florida_

Tagged colonies of _G. ventalina_ were variable between 2005 and 2007, especially with respect to $\delta^{15}\text{N}$. There was no difference between Carysfort and Pickles reef, suggesting that these two reefs have similar N sources, and are therefore potential replicates for N source monitoring. The isotope ratios for both C and N vary in a similar though reversed pattern, which may or may not be due to land-based activities. Ultimately, these data are important for showing that variability is observed from year to year, and thus, long-term monitoring of trends in isotope ratios could be a powerful tool for assessing the impact of coastal development, population growth, and environmental remediation efforts, such as the recent sewage treatment plant upgrades in Key West, FL which terminated the operation of a sewage outfall.

_Power analysis_

Using power analysis we show that using gorgonian corals to detect differences between sites as small as 0.3 ‰ may be possible with < 10 samples per site. Detecting statistical significance below this threshold would be limited by instrument precision.
In general, gorgonians require much smaller sample sizes than macroalgae to have the power to detect differences of 1.0 ‰, a threshold value suggested by Lapointe et al. (2004) and Heikoop et al. (1998) as indicative of sewage derived N pollution between pristine and impacted sites. We used power analyses to determine the utility of gorgonian corals for isotope studies in comparison to other tropical benthic marine biota. Lapointe et al. (2005) quantified \( \delta^{15}\text{N} \) of bloom macroalgae off the coast of Southern Florida from which we obtained a conservative, site-specific standard deviation in \( \delta^{15}\text{N} \) of 1.27 ‰. \textit{G. ventalina} from Carysfort reef had a standard deviation of 0.24 ‰ for all years sampled. To have equivalent power (0.99) to detect a difference of 1.0 ‰ between sites you would need 4 sea fans, or 32 macroalgae samples. Low variability among individuals within a specific location is a desirable characteristic of gorgonians, which require fewer samples to obtain results relative to other functional groups of benthic marine organisms.

Conclusions

Shallow-water gorgonian corals are indeed powerful and precise recorders for stable isotope analysis (Risk, 2009, Risk et al., 2009). Collecting and processing gorgonian corals is uncomplicated, requiring collection of small portions of the colony edge, air-drying, and minimal sample handling prior to analysis. The latter is especially true for \( \delta^{15}\text{N} \) determination. C isotope analysis of gorgonians is vulnerable to the confounding effects of lipids and sclerites, though removal of these components via lipid extraction and acidification, respectively, has negligible impacts on \( \delta^{15}\text{N} \) allowing for dual-isotope determinations on single samples. Field sampling designs should consider 1) appropriate species for analysis, with the consideration that sea rods are more heterotrophic than sea plumes and fans and 2) light-mediated isotope effects that can
confound data interpretation between sites that differ in depth or turbidity, although these effects are small in comparison to the impacts of large sources of anthropogenic pollution (Baker et al. in preparation). These ubiquitous corals show significant change in isotope ratios year to year, have higher power to detect differences spatially and temporally than macroalgae, and are therefore an excellent target for monitoring changes in C and N sources to coral reefs.
REFERENCES


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CHAPTER 2

NITROGEN ISOTOPE RATIOS OF THE CALCAREOUS ALGA *HALIMEDA SPP.*; SPATIAL AND INTRA-ANNUAL VARIABILITY

AND

THE RELATIONSHIP WITH WATER QUALITY IN THE FLORIDA KEYS NATIONAL MARINE SANCTUARY

Abstract

Stable isotope ratios of nitrogen ($\delta^{15}N$) contain information about N sources and are frequently used as an integrated record of pollution in coastal marine ecosystems. However, few studies have quantified natural variations in $\delta^{15}N$ over large spatial and temporal scales. Understanding this variability, and how environmental factors such as water column chemistry affect $\delta^{15}N$ is crucial for interpreting isotope data with the goal of identifying sources of anthropogenic N pollution. In this paper we present evidence that $\delta^{15}N$ of primary producers can be used to observe spatial and temporal change in N sources, but cannot be used reliably as an integrator of concentrations of water column N in a changing N seascape. To test the hypothesis that water quality parameters affect isotope ratios of primary producers, we analyzed the $\delta^{15}N$ of macroalgae sampled from 30 sites within the Florida Keys National Marine Sanctuary (FKNMS) that are well characterized by the Southeast Environmental Research Center’s Water Quality Monitoring Network (SERC-WQMN). Here, we show that $\delta^{15}N$ values from samples of calcareous algae collected over 4 seasons and across $\sim4,000$ km$^2$ span a range of 11.5 ‰, reflecting a highly dynamic system with respect to N sources. Our data support the hypothesis that $^{15}N$ enriched groundwater is
affecting nearshore habitats in the Upper Keys region as evidenced by elevated $\delta^{15}$N values. Furthermore, $\delta^{15}$N increased in the wet season at sites alongshore, supporting previous observations of land-based inputs of sewage associated with rainfall. Our main goal was to test the hypothesis that water quality parameters are correlated with $\delta^{15}$N values. Although regression analyses revealed significant correlations between $\delta^{15}$N and seven long-term average water quality values, these analyses are misleading because many parameters co-vary. In fact, a comparison between two random-effects models with and without water quality parameters failed to support the hypothesis that water quality factors influence variation in $\delta^{15}$N, leading to our conclusion that $\delta^{15}$N is not significantly affected by water quality.

**Introduction**

In recent years, the transition of coral reefs to algal-dominated barrens is a common observation in the Florida Keys and throughout the Caribbean. As the third largest barrier reef in the world, the South Florida reefs have seen dramatic losses of coral, up to 85% for some major species (Lidz & Hallock, 2000, Somerfield *et al.*, 2008). This decline has been linked to chronic nutrient pollution from local sewage disposal and upstate agricultural runoff into Florida Bay (Lapointe *et al.*, 2004). Wastewater and agricultural run-off, which are often high in nitrogen (N) and phosphate (P) are associated with algal blooms (Lapointe, 1997, Lapointe *et al.*, 2004) and have been implicated in increasing disease severity in corals (Baker *et al.*, 2007, Bruno *et al.*, 2003, Voss & Richardson, 2006) and seagrass decline (Waycott *et al.*, 2009). The human population in the Keys is especially dense with up to 800 persons per km$^2$. Moreover, south Florida operates 6 sewage outfalls, which collectively release nearly 400 million gallons of partially treated wastewater offshore per day (Koopman *et al.*, 2004).
This quantity does not include the sewage contribution from Monroe County’s ~26,000 on-site septic systems which leach nitrogen directly into canals, seagrass beds, and coral reefs (Corbett et al., 2000, Corbett et al., 1999). A recent study concluded that all near-shore habitats less than 30m in depth are dominated by sewage-derived nitrogen in the FKNMS (Lapointe et al., 2004).

In 1995, the SERC-WQMN was established in an effort to monitor change and define acceptable baselines for environmental quality in the FKNMS. Quarterly measures of water quality have revealed heterogeneity among sites with respect to nutrient loads, and more importantly, system-wide increases in nitrate (NO$_3^-$) and total phosphate (TP), particularly near developed shorelines; a sign of sewage pollution (Jones & Boyer, 2000). While concentrations are valuable for documenting long-term and large-scale geographic variation in water quality, they lack the temporal resolution needed to record important episodic N flux (Leichter et al., 1996). For instance, upwelling events are an important albeit ephemeral source of N to reefs in the FKNMS. On Conch reef, episodic upwelling increases N concentrations up to 40x ambient levels, and quickly subsides through rapid biotic assimilation (Leichter et al., 1996). More importantly, conventional water quality assessments are unable to distinguish between natural and anthropogenic sources, a critical gap for assessing the impacts of development, and conversely, documenting the return to natural baselines following remediation efforts.

Over the last decade the application of stable isotope analyses of benthic marine organisms has increased in response to the problem of unprecedented N pollution from land (Howarth et al., 2000, Vitousek et al., 1997). Isotope values reflect the origin of N, and anthropogenic sources are generally distinguishable from natural sources of N.
Stable isotope values provide an integrated record of the relative contributions of nitrogen sources in the marine environment. Analysis of the ratio of $^{15}\text{N}/^{14}\text{N}$ (expressed as $\delta^{15}\text{N}$ in units ‰) can detect the presence of anthropogenic N in the tissues of primary producers (McClelland & Valiela, 1998, Risk, 2009, Umezawa et al., 2002). Agricultural fertilizers and atmospheric N deposition from combustion of biomass and fossil fuels have a range of possible $\delta^{15}\text{N}$ values, though on average tend to be < 0 ‰, while human and animal wastes tend to be enriched in $^{15}\text{N}$ (> 8 ‰) primarily from microbial denitrification and losses of $^{14}\text{N}$ via ammonia volatization (Heaton, 1986, Paerl & Fogel, 1994). As an organism assimilates N from these sources, they will become isotopically similar to that source. Signals of anthropogenic pollution can be very clear when single sources are involved (Baker et al. in preparation, (Risk & Erdmann, 2000). However, in highly polluted systems where multiple sources are mixed and integrated by the marine food web, the ability to distinguish anthropogenic sources becomes difficult (Lapointe et al., 2004, Risk, 2009). Quantifying isotope ratios over space and time can ameliorate this difficulty by detecting increasing proportions of anthropogenic nutrients over time, and relative differences in N sources between locations, respectively. However, we must first assess the natural seasonal and spatial variability within a study system before drawing conclusions about ecosystem health (Campbell & Fourquean, 2009, Fourquean et al., 2005, Fourquean et al., 2007).

Marine primary producers enriched in $^{15}\text{N}$ are a useful record for sewage pollution (Costanzo et al., 2005, Lapointe et al., 2004, Mutchler et al., 2007, Risk et al., 2009, McClelland & Valiela, 1998, Valiela et al., 1997). Algae are common targets for sampling, as they are widespread and easy to collect and store. More importantly, algae assimilate inorganic N, primarily ammonium (Lapointe et al., 2004), and
therefore effectively record inorganic N pollution in the environment. Sewage effluents contain high concentrations of nitrogen, primarily in the form of nitrate and organic N (Griggs et al., 2003). Specimens of the red fleshy alga Catenella distributed throughout Moreton Bay, Australia recorded a decline in sewage-derived nitrogen inputs after a treatment plant upgrade (Costanzo et al., 2005). These algal recorders were deployed for only 4 days before retrieval and analysis. This method was effective because fleshy macroalgae have high rates of N uptake and therefore reflect N sources assimilated on the order of days (Gartner et al., 2002). Gartner et al. (2002) demonstrated that the leathery kelp Ecklonia radiata, having a low rate of N uptake, showed a slow $\delta^{15}$N response compared to fleshy algae when cultured with enriched $^{15}$N sewage effluent. For this reason, slow growing species are better longer-term integrators of N sources and offer a weighted average of environmental N sources on the scale of weeks to months.

Similarly, calcareous species grow slowly, are long-lived, do not form blooms, are spatially discrete (non-drifting), and their growth is not increased substantially with pulses of nutrients (Delgado & Lapointe, 1994). N limits the productivity of Halimeda opuntia year-round in the Florida Keys, even under eutrophic conditions (Delgado & Lapointe, 1994) which means that $\delta^{15}$N of the algae should closely approximate the sources assimilated with little fractionation. Therefore, calcareous algae are a good integrator of N sources on the order of weeks to months (Gartner et al., 2002, Umezawa et al., 2002). Halimeda species are ubiquitous in the FKNMS making them an ideal target for examining spatial and temporal trends in isotope ratios.
Interpretation of isotopic data can be confounded by fractionations associated with natural environmental phenomena, and lead to erroneous conclusions that anthropogenic N sources are in the environment. For producers, fractionations associated with N limitation and rates of photosynthesis are likely. When N is not limiting to growth, enzymatic reactions have a higher affinity for the $^{14}$N isotope. Conversely, under N-limited conditions this fractionation is reduced by the need to sustain metabolism. Baker et al. (2007) found that $\delta^{15}$N of the gorgonian coral *Gorgonia ventalina* was highest in the Marquesas, a site far removed from human influence in the FKNMS and characterized by chronically low N concentrations. One possible explanation for this observation is that N was limiting productivity at this site, reducing fractionation between the gorgonian and the source $\delta^{15}$N and consequently, driving these values higher relative to the $\delta^{15}$N of individuals sampled from sites where N was not limiting. In non-limiting conditions, increasing proportions of anthropogenic N can be detected. Ward-Paige et al. (2005) found that high $\delta^{15}$N from gorgonian corals were positively correlated with total N (TN) concentrations at sites in the FKNMS. This result could only come about if there was an increasing proportion of sewage contributing to the total N pool (McClelland & Valiela, 1998). However, $\delta^{15}$N of fleshy algae in the Florida Keys is driven by seasonal transport of N from disparate land-based sources, and not necessarily dependant on N concentrations (Lapointe et al., 2004). Similar fractionations can occur as rates of photosynthesis are affected by light intensity. As light increases, both photosynthetic rates and the internal demand for N increase which reduces fractionation between the source and the organism. Interestingly, light-mediated isotope fractionations have been reported for corals (Heikoop et al., 1998), but not algae. Quantifying the fractionation factors associated with environmental gradients of nutrients and light availability is a critical
gap in our understanding of $\delta^{15}$N dynamics in coastal marine systems, and should be understood before conclusions of anthropogenic pollution are drawn.

Here, we use calcareous macroalgae to monitor nitrogen isotope values in the FKNMS. Our first goal was to determine the natural variability in $\delta^{15}$N over space and time. Spatial variability was estimated by comparing regions and habitat types with the hypothesis that regions with large human populations (Upper and Lower Keys) and sites nearshore and thus closer to human development are impacted by sewage derived N. Temporal variability was estimated by sampling quarterly within a year to test the hypothesis that $\delta^{15}$N varies with a) productivity, and b) rainfall during the wet season which flushes sewage and agricultural fertilizers into Atlantic and Florida Bay, respectively. Our second goal was to test the hypothesis that $\delta^{15}$N of macroalgae is correlated with long-term water quality values, specifically concentrations of N and P, N:P, and other metrics for productivity from the same locations to reveal factors that may drive isotopic fractionations in the environment.

**Methods**

The Florida Keys is a series of small islands, forming a ~250 km archipelago off the southern tip of Florida. The islands are atop 2 main geological formations, the Key Largo Limestone, and the Miami Limestone (Hoffmeister & Multer, 1968). The Key Largo Limestone underlies the Upper and Middle Keys regions (Fig. 2.1) and consists of an ancient framework of coral skeletons, which in conjunction with weathering, creates a highly porous substrate through which groundwater is easily conducted (Halley et al., 1997). In comparison, the Miami Limestone that underlies the Lower Keys is comprised of small calcareous sediments of various origins and is less
permeable to water. The hydrodynamics of the region are primarily driven by tides, and creates a disparity in the water height between Florida Bay and the Atlantic sides of the Keys (Corbett et al., 2000, Halley et al., 1997). This difference generates a net flux of Bay and ground waters towards the ocean (Halley et al., 1997). The Upper, Middle, and Lower Keys regions contain three habitat types; 1) offshore reefs which are characterized by hard-bottomed coral communities, 2) channel sites, which are typically sandy and dominated by seagrasses and large sponges, and 3) alongshore sites, which may be hard bottomed or sandy/silty areas with a various benthic communities, though mostly seagrass dominated (Fourquarean et al., 2005, Jones & Boyer, 2000). In contrast, the Sluiceway and Backcountry are regions within Florida Bay. Sites within these regions are very shallow, with sandy and muddy substrates that support large communities of seagrass and algae.
Field collections were conducted in December 2007, and in March, June, and September of 2008. We sampled 30 sites within the FKNMS using SCUBA at depths ranging from 2 – 14 m (Fig. 2.1). The sites were previously selected for a long-term seagrass monitoring program (Fourquean et al., 2005). At each location, we sampled up to 5 whole calcareous macroalgae of the genus *Halimeda* (*n* = 280) and *Penicillus* (*n* = 12). These genera were found to have indistinguishable $\delta^{15}$N (Student’s t-test, $t = -0.29$, $p$ (two-tailed) = 0.77). For both genera, the entire alga was sampled and held on ice prior to rinsing to remove epiphytic growth and oven-drying overnight.

Each sampled site corresponds with a station monitored by the SERC-WQMN, where a suite of chemical and physical properties has been quantified on a quarterly basis since 1995 (Jones & Boyer, 2000). To test the hypothesis that water quality parameters influence isotope ratios, we targeted several factors for our analysis (all units in mM unless otherwise noted): total N (TN), total organic N (TON), dissolved inorganic N (DIN), soluble reactive phosphate (SRP), dissolved inorganic N to total phosphate (DIN:TP, ratio), total organic carbon (TOC), temperature (TEMP, degrees C), salinity (SAL, ppt), and dissolved oxygen (DO, % sat.). Long-term mean water quality values were calculated for each site from 1995 – 2008 using the more comprehensive surface dataset.

*Stable isotope analyses*

The proximal thallus of each plant was removed and homogenized by grinding using a mortar and pestle. The resulting powder was weighed into 4 x 6 mm tin capsules, and combusted in a Carlo-Erba elemental analyzer coupled to a Finnegem-MAT Delta Plus isotope ratio mass spectrometer. Reported $\delta^{15}$N values are relative to atmospheric N$_2$.  

Precision for $\delta^{15}$N was quantified with an in-house standard (‘bcbg’) calibrated against IAEA international standards, which was better than 0.12 ‰.

**Statistical analysis**

Prior to analysis, all data were screened for normality and homoscedasticity using Shapiro-Wilk and Levene’s tests, respectively. To test for spatial variation (i.e. between regions and habitat types) we used ANOVA, and where overall effects were detected we used post-hoc pairwise t-tests to determine significant differences between groups. Independent linear regressions were used to characterize the relationship between $\delta^{15}$N and long-term water quality averages. To control for multiple tests, we applied a Sequential Bonferroni correction (Rice, 1989). We also used a random-effects model to test the hypothesis that macroalgae $\delta^{15}$N values are affected by water quality parameters and seasonal (quarterly) variation (fixed factors), and site (random factor). We compared this model to one constructed without water quality parameters. Using a chi-squared analysis on the difference between the likelihood estimates of the models, we assessed the relevance of water quality as a whole on our conclusions. All statistical analyses were conducted using JMP v.8.0 (SAS, Cary, NC).

**Results**

Overall $\delta^{15}$N values ranged from -6.9 to 4.6 ‰ with a mean ($\pm$ SD) of 1.5 ± 1.8 ‰ ($n = 292$; Fig. 2.2). The range of values regionally (7.5 ‰) was equivalent to the range of values over time (8.4 ‰). These data were negatively skewed (skewness = -1.6), and therefore did not approximate a normal distribution (Shapiro-Wilk’s test, $p > 0.05$) and
were not homoscedastic (Levene’s test, $p < 0.05$). The data were normalized to positive, non-zero values by addition of the absolute value of the minimum (6.9 ‰). The resulting sums were transformed using a Box-Cox transformation. The transformed values were not normally distributed, though the skewness was reduced (-0.6) and the transformed values were homogeneous with respect to variance (Levene’s test, $p = 0.16$). All subsequent statistical tests were performed using these transformed data.

Figure 2.2. Frequency distribution of N isotope ratios of macroalgae from the FKNMS.
Spatial variation:

There was a significant effect of region on $\delta^{15}\text{N}$ (ANOVA, $F_{4,292} = 15.89$, $p < 0.001$; Fig. 2.3). Samples from the Lower Keys had the highest $\delta^{15}\text{N}$ values (mean = 2.19 ‰) and were not statistically different from the Upper Keys (2.18 ‰). In comparison, samples from the Sluiceway (1.31 ‰) and Middle Keys (1.29 ‰) had significantly lower $\delta^{15}\text{N}$, though these regions were enriched relative to the Backcountry (-0.26 ‰; post-hoc pairwise Student’s t-test, all $p < 0.05$).

Figure 2.3. Variation in mean $\delta^{15}\text{N}$ (± SE) of calcareous macroalgae sampled throughout five major regions of the FKNMS including sites in the Atlantic (Upper, Middle, and Lower Keys) and Florida Bay (Backcountry, Sluiceway) from 4 seasonal collections throughout 2008.

Within the Atlantic sites, we were able to compare along-shore, channel, and reef habitats as defined by the SERC-WQMN. Within each region, these three habitat
types represent a nearshore to offshore gradient. In the Upper Keys, there was a significant effect of habitat type on $\delta^{15}$N (ANOVA, $F_{2,64} = 10.49, p < 0.0001$; Fig. 2.3) with the highest values alongshore (2.53 ‰), slightly lower values among channel sites (2.45 ‰), and significantly lower $\delta^{15}$N among reef sites (1.3 ‰; Student’s post-hoc t-test $p < 0.05$). This trend was reversed in the Lower Keys, where $\delta^{15}$N increased from alongshore to the reef (ANOVA, $F_{2,77} = 9.03, p = 0.0003$; Fig. 2.4). Here, macroalgae from reef habitats were significantly enriched (2.85 ‰), relative to the channel (2.13 ‰) and alongshore sites (1.55 ‰). There was no effect of habitat type on $\delta^{15}$N in the Middle Keys (ANOVA, $F_{2,55} = 0.57, p = 0.57$).

Figure 2.4. Mean $\delta^{15}$N (± SE) of macroalgae samples along 3 alongshore-channel-reef transects on the Atlantic side of the FKNMS (as defined by Boyer et al., factor “ZSEG”). Asterisks denote significant differences between areas at $\alpha = 0.05$. 

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<td>2.85</td>
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</table>
Temporal variation

Keys-wide, samples collected in the winter (Q01) were 0.6 ‰ depleted relative to the annual mean $\delta^{15}$N of 1.55 ‰ (ANOVA, $F_{3,292} = 6.99, p = 0.0001$, Student’s post-hoc t-test, $p < 0.05$; Fig. 2.3). This pattern was driven primarily by samples from alongshore and reef habitats that had relatively depleted values in the winter. Low winter values were found in all regions with the exception of the Backcountry, which had lowest $\delta^{15}$N values in the spring (Q02). The highest $\delta^{15}$N values were typically observed in the summer (Q03), particularly in alongshore and reef habitats.

Figure 2.5. Mean $\delta^{15}$N (± SE) of macroalgae samples collected in the wet and dry season from five regions in the FKNMS. Asterisk denotes significant difference between seasons at $\alpha = 0.05$. 
South Florida has a distinct rainy (January – April) and dry season (May – September; (Lapointe et al., 2004). Therefore our first and second quarterly samples can be loosely categorized as wet season, and the third and fourth quarters dry season. Atlantic regions had higher $\delta^{15}$N in the wet season, relative to samples taken in the dry season (Fig. 2.5). This observation was reversed in the Backcountry and Sluiceway where samples from the wet season had slightly lower $\delta^{15}$N values. These seasonal differences were not statistically significant with the exception of the Upper Keys where samples taken in the wet season were 0.6 ‰ higher than the dry season ($n = 65, t = 2.1, p$ (one-tailed) = 0.02). We conducted a similar analysis by habitat type, which showed that alongshore sites were significantly enriched in $^{15}$N during the wet season, by 0.9 ‰ ($t$-test, $n = 85, t = 3.03, p$ (one-tailed) = 0.003; Fig. 2.6).

**Figure 2.6.** Mean $\delta^{15}$N ($\pm$ SE) from five habitat types (as defined by Boyer et al.) sampled in the wet and dry seasons. Asterisk denotes significant difference between seasons at $\alpha = 0.05$. 
Bivariate fits of $\delta^{15}$N as a function of water quality parameters frequently produced significant trends with very high variability (Table 2.1). Seven of the eleven correlations were highly significant, even when alpha was adjusted for multiple comparisons ($\alpha = 0.05/11 = 0.0045$). Interestingly, $\delta^{15}$N was not correlated with DIN or SRP, variables which should strongly influence algal growth. The correlation between DO and $\delta^{15}$N was strongest, though highly variable, accounting for only 11% of the variation in $\delta^{15}$N.

Table 2.1. Results of correlation analyses with site-specific macroalgal $\delta^{15}$N as a function of select water quality parameters (long-term quarterly means from 1995 – 2008). Rows in bold indicate significance after a Sequential Bonferroni correction was applied (table-wide $\alpha = 0.004$).

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<td>0.00</td>
<td>0.41</td>
</tr>
<tr>
<td>SRP</td>
<td>-24.9</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
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<td>0.0002</td>
</tr>
<tr>
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<td>0.07</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>TOC</td>
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<td>0.03</td>
<td>0.006</td>
</tr>
<tr>
<td>TON</td>
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<td>0.07</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>TP</td>
<td>-8.38</td>
<td>0.09</td>
<td>$&lt;0.0001$</td>
</tr>
</tbody>
</table>

To better assess the relative explanatory power of spatial factors versus water quality parameters, we used a random-effects model, which accounted for 78% of the variation in $\delta^{15}$N, with a significant effect of season sampled (QUARTER, $p = 0.011$). As a random factor, site accounted for 30% of the random variability, while site * quarter accounted for 38%. The inclusion of water quality parameters again illustrated
a significant effect of quarter ($F_{3,292} = 3.00, p = 0.036$) and DO on $\delta^{15}N$ ($F_{1,292} = 6.81, p = 0.011$). However, the water quality inclusive model was not significantly different than the model using only spatial and temporal parameters (chi-squared analysis on the difference in the -2*log likelihood estimate, $df = 10$, $C^2\text{-crit} = 18.10, p > 0.05$) suggesting that water quality overall had no impact on the model outcome, and did not significantly increase the explained variance in $\delta^{15}N$.

**Discussion**

Nitrogen isotope ratios of macroalgae in the FKNMS were highly variable, ranging on average 7.5 ‰ over ~4,000 km$^2$, 8.4 ‰ over 4 quarterly measurements, and 11.5 ‰ overall (Fig. 2.2). The variation across regions was equivalent to the variability between quarters, suggesting that temporal and spatial factors are equally important when sampling for stable isotope analyses. The large variability in $\delta^{15}N$ strongly suggests that the sources of N integrated by algae are highly varied over space and time. Important sources of N in the FKNMS are sewage and residual N from microbial nitrification and denitrification (Corbett et al., 2000), N fixation (Wada & Hattori, 1976), agricultural fertilizers (Heaton, 1986), upwelling (Leichter et al., 2007), and atmospheric deposition (Paerl & Fogel, 1994). Although the relative importance of any individual source is debatable, using $\delta^{15}N$ analysis we can monitor over time increases in the proportion of those anthropogenic sources that have large differences in $\delta^{15}N$ relative to natural sources. The sum of these sources, with respect to a given N species (i.e. NO$_3^-$, NH$_4^+$, etc.) can be quantified by measuring the concentration of these species in the water column, though actual water column concentrations are often underestimated due to rapid biotic assimilation (Leichter et al., 1996). The relative proportions of these sources, and the fractionations associated with their
movement through the environment and during assimilation are reflected in the isotope ratio. As these isotope ratios within sites and regions are changing through time, we can infer that the sources of N, and/or the conditions that fractionate N isotopes are also changing. Given that $\delta^{15}$N was so variable in this study, it is important to discuss patterns observed with respect to space and time.

Our study revealed that algae sampled within the Upper and Lower Keys regions were enriched in $^{15}$N relative to the other regions, particularly in the summer when productivity is highest, and thus, producer $\delta^{15}$N closely reflects N sources (Fig. 2.3). Primary producers sampled from the Upper Keys have been previously identified as having enriched $\delta^{15}$N. Corbett et al. (1999) documented high $\delta^{15}$N values (6 - 13 ‰) in macroalgae and seagrass tissues sampled from the Florida Bay side of the Upper Keys. Our data, while not as highly enriched in $^{15}$N were the highest in this region. The difference between our results and those of Corbett et al. (1999) could be due to the fact that our Upper Keys sampling locations were on the Atlantic side of the Keys where contaminated groundwater is rapidly diluted by the ocean. We also observed higher $\delta^{15}$N values in nearshore habitats in this region, and these values were progressively lower in channel sites, and lower still on reef sites (Fig. 2.4). The decline in $\delta^{15}$N along this near- to offshore gradient suggests a relatively enriched N source alongshore, possibly sewage contaminated groundwater that is diluted by seawater with increasing distance away from the source (Corbett et al., 1999, Griggs et al., 2003). Interestingly, this observation was entirely reversed in the Lower Keys, suggesting that either; 1) sites alongshore in the Lower Keys are not impacted by a similar enriched N groundwater source, or 2) offshore upwelling is more important in this region (Fig. 2.4). $\delta^{15}$N values for deepwater NO$_3^-$ offshore of the FKNMS are between ~ 4.5 and 5.5 ‰. Thus in the absence of an enriched N source from land it is
possible that upwelling could drive isotopic enrichments offshore if algae are assimilating relatively more upwelled NO$_3^-$ . However, whether algae utilize upwelled NO$_3^-$ has been contested as NH$_4^+$ is preferentially assimilated at a faster rate than NO$_3^-$, and, simultaneously, inhibits NO$_3^-$ uptake, though this has not been tested in *Halimeda* (Lapointe *et al.*, 2005, Leichter *et al.*, 2007). Lapointe *et al.* (2004) offer the alternative hypothesis that intrusions of NH$_4^+$ from sewage contaminated groundwater reaches offshore reefs, particularly in the Lower Keys where upwelling events are rare.

The Middle Keys on the other hand, showed the highest variability in $\delta^{15}$N, and no significant change along the near- to offshore transect (Fig. 2.4). The Middle Keys sites are most connected to Florida Bay, having several large expanses between landmasses where large inflows from the Bay are evident. Samples from the Middle Keys were among the lowest of the Atlantic-side regions. This is likely due to low $\delta^{15}$N sources entering from Florida Bay, possibly from agricultural fertilizers from the North, or from relatively higher rates of N fixation in the shallow anoxic areas of Florida Bay. However, the results from the Middle Keys were also seasonally noisy with highest variability in the winter and spring. Increased noise in $\delta^{15}$N would be expected in this region where low $\delta^{15}$N sources from Florida Bay are mixed with $^{15}$N enriched sources from land leaching onto these sites and, potentially, upwelling in reef habitats. Mixing of natural and anthropogenic sources can produce a broad spectrum of $\delta^{15}$N values, and thus confound interpretation of $\delta^{15}$N from organisms that have integrated those sources (Risk *et al.*, 2009).

Sites within Florida Bay had the lowest mean $\delta^{15}$N. The Backcountry, particularly in the spring, had the lowest $\delta^{15}$N observed in this study averaging -3.3 ‰ (Fig. 2.3 &
2.5). These very low isotope ratios were driven by samples collected from the western-most sites in Florida Bay (station 305 and 309). Corbett et al. also observed low $\delta^{15}$N of primary producers in the western-most sites of Florida Bay sampled in their study, compared to relatively enriched values from samples from the northeast. They hypothesized that a low $\delta^{15}$N source from the Gulf of Mexico is progressively denitrified towards the Keys, though they did not explain why the suspected source from the Gulf was relatively depleted originally. N fixation, having no fractionation between the source (atmospheric N$_2$) and sink (organic N) is a likely source of low $\delta^{15}$N values (Wada & Hattori, 1976). In non-limiting conditions an organism can discriminate against the heavy isotope, and thus become depleted relative to the source. The average $\delta^{15}$N of algae from the Backcountry is within $\sim 1.0$ ‰ of the known range for fixed N sources (Wada & Hattori, 1976), and therefore could be an important source of N in this region.

There was a general trend of increasing $\delta^{15}$N towards the summer, and low $\delta^{15}$N in the winter (Fig. 2.3). Fourquarean et al. (2005) reported a similar pattern in the seagrass *Thalassia testudinum*, and hypothesized that during the seasons of highest productivity, there is reduced isotopic fractionation associated with uptake and assimilation due to high demand for N, reduces the size of the local N pool. Under these conditions $^{15}$N discrimination is lessened, thus isotopically, the plant $\delta^{15}$N more closely approximates the source. While seasonal variation is important to understand for designing isotope experiments or monitoring, it can be said that sampling in the season of highest productivity gives the best information on the sources as the effects of fractionation are minimal. Much like seagrasses, the *Halimeda* species sampled for this study are rhizobial, having rootlike bulbs or holdfasts that extend into the sediments. These structures are known to assimilate nutrients from sediment pore
waters (McGlathery et al., 1992, Williams, 1984). Thus, much like seagrasses, these algae can assimilate N from 2 environmental pools, the water column and sediments. The origin and isotopic transformations of N within these pools differ, and may account for the high variability observed in this study. Regardless, algae $\delta^{15}N$ appears to increase toward the summer months, and then decline towards the winter, though not in all regions.

We found that spatial and temporal factors alone explained the most variation in $\delta^{15}N$, and had equal variability. We found no evidence that water quality parameters, especially the concentrations of N in the water column and N to P ratios, have any influence on $\delta^{15}N$ of macroalgae. This is not surprising, as isotope ratios are tracers for N sources, and are independent of N concentrations. In other words, it doesn’t depend on how much N there is, just where it comes from. Rhizobial algae and other rooted marine plants may be poor candidates for $\delta^{15}N$ monitoring as they are recording both sediment and water column N sources (McGlathery et al., 1992, Williams, 1984). Sediment microbial transformations of $\delta^{15}N$ and subsequent assimilation by an alga could produce a disconnect between $\delta^{15}N$ of the alga and water column chemistry.

Biological integrators that record N sources over longer timescales (i.e. turnover/growth over months to years) may overcome the issue of noise from small-scale sources. Long-term water quality records for TN were found to correlate with gorgonian coral (Ward-Paige et al., 2005a) and sponge $\delta^{15}N$ in the FKNMS (Ward-Paige et al., 2005b). Both corals and sponges represent longer-term integrators of N sources and are probably not as sensitive to N from pore waters. However, closer inspection of their data reveals that not only TN, but also thirteen additional parameters were correlated with $\delta^{15}N$, at times with stronger correlations than reported.
for TN. Similarly, we show that despite high variability, seven of eleven water quality parameters were correlated with algae $\delta^{15}N$, even when a conservative threshold for significance was applied (Table 2.1). Ward-Paige et al. did not examine other water quality parameters, which would have necessitated more rigorous criteria for assessing statistical significance (Rice, 1989). However, they also gave no a priori reasoning for why TN was selected as the only water quality parameter suspected to drive $\delta^{15}N$. Given that elevated $\delta^{15}N$ at sewage impacted sites that also have high ammonium concentrations there are reasonable qualitative conclusions to be made, though the correlation here is linked to a gradient of environmental impacts and not due to a causative link between $\delta^{15}N$ and concentrations. More importantly, these authors did not consider the implications of their conclusions; a correlation between the concentrations of N species (or sum of those species) found with $\delta^{15}N$ would suggest that that the N pool is entirely composed of one source. Even this reasoning is tenuous, as we would expect this relationship to break down along a continuum of nutrient limitation as isotopic fractionations change (Umezawa et al., 2008, Umezawa et al., 2002). For example, in N limiting conditions, discrimination against the $^{15}N$ isotope is reduced in order to sustain metabolism, thus $\delta^{15}N$ could be negatively correlated with N concentrations in limiting conditions. Baker et al. (2007) evoked limitation as an explanation as to why the highest $\delta^{15}N$ values in a sampling of gorgonian corals were recorded from a site with the lowest N concentrations and considerably removed from human development. Furthermore, samples of the gorgonian Pterogorgia sp. and the sponges Ircinia sp. and Amphimedon sp. collected during this study along with the algae samples produced similar results in the random-effects model comparison (data not shown). We therefore conclude that in this system there is not sufficient evidence for a causative link between $\delta^{15}N$ and water quality among short- to long-term biological integrators.
In the Florida Keys we have shown that $\delta^{15}$N values in common calcareous macroalgae vary widely among sites and quarterly samplings, emphasizing the importance of exercising caution in interpreting snapshot measures of $\delta^{15}$N from few locations when the potential background variability is high. Consider that $\delta^{15}$N increases on average by 3.4 ‰ between trophic levels due to retention of $^{15}$N within consumers (McCutchan et al., 2003, Minagawa & Wada, 1984). Thus, the range of $\delta^{15}$N we report here is similar in magnitude to the difference expected between a primary producer and a tertiary consumer within the same food web, even though there is no trophic enrichment expected in algae. It is likely that enriched values observed in our study are a result of an increased proportion of sewage N in those environments, as the highest $\delta^{15}$N values were recorded in the wet season, particularly in alongshore habitats of the Upper Keys; an observation that has been reported previously (Corbett et al., 2000, Kruczynski et al., 2002). However, our results are not supportive of suggested threshold offered by Lapointe et al. (2004); that $\delta^{15}$N values greater than 3.0 ‰ in primary producers is evidence of sewage derived-N. Using this baseline, these authors concluded that all sites <30 m depth in the FKNMS are dominated by sewage N. We found no sites where average $\delta^{15}$N exceeded 3.0 ‰ and among individual samples only 53/292 samples (18%) were > 3.0 ‰, which is not strong support for widespread sewage inputs as defined by Lapointe et al. (2004). One potential difference between our studies is that we used calcareous rather than fleshy macroalgae for analysis. Many fleshy algae have high rates of N uptake and therefore have the potential to form algal blooms particularly following episodic stormwater run-off, which causes spikes in anthropogenic N inputs (Delgado & Lapointe, 1994, Lapointe et al., 2005, Lapointe & Matzie, 1996). As such, we would expect that ephemeral fleshy algae capitalizing on anthropogenic sources would have isotope
ratios that reflect those sources more frequently. On the other hand, calcareous algae, having slower growth rates and N uptake rates are less likely to be “biased” towards episodic inputs of anthropogenic sources, as these inputs do not affect the abundance of these species. While useful for managers and environmental monitoring, threshold values for perceived anthropogenic inputs should be qualified as species-specific.

Future research on using stable isotopes for source tracking should focus on the environmental constraints on primary productivity and how the physiological ecology of marine plants mediates isotope ratios, particularly for N. While we found no impact on the relative availability of N to P on δ₁⁵N in this study, it is potentially confounding that under N limitation isotopic enrichment could lead to the erroneous conclusion that sewage N is present. Furthermore, the effects of light gradients, whether by chronic turbidity or attenuation over depth, on algal δ₁⁵N is likely to be important, as has been documented in corals (Heikoop et al., 1998), and is worthy of investigation. Recently, Campbell and Fourquean (2009) reported a positive relationship between δ₁⁵N and depth for the seagrasses Halodule wrightii and Syringodium filiforme, but not in Thalassia testudinum. The highest isotope values were reported at the deepest sites, a pattern that could be driven by N limitation. This observation is counter to what has been observed in hard corals (Heikoop et al., 1998), and gorgonians (Baker, et al., in preparation). Therefore, future research should focus on quantifying the effects of light and nutrient limitation on isotope ratios of macroalgal species. Finally, studies that have failed to detect a correlation between water column concentrations and other metrics for water quality should report these negative results, to reinforce the hypothesis that N isotope ratios are affected by N sources, and not the size of the N pool.
Acknowledgements

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CHAPTER 3

GORGONIAN CORALS PROVIDE A STABLE ISOTOPE BASELINE FOR ASSESSING SEWAGE POLLUTION IN THE MEXICAN CARIBBEAN

Abstract

Identifying and monitoring sources of sewage pollution is a critical need for conserving and managing the impacts of development on coastal marine ecosystems. Geochemical analyses of marine invertebrates are increasingly used as an integrated record for sewage. The ratio of the stable isotopes of nitrogen (N; $^{15}$N/$^{14}$N or $\delta^{15}$N) is useful for tracing sources of sewage-derived N, as human and animal wastes tend to be enriched in the heavy isotope, $^{15}$N. I quantified the $\delta^{15}$N values in the common Caribbean sea fan *Gorgonia ventalina* (Class: Anthozoa, Family: Gorgoniidae) to test the hypothesis that sewage derived N inputs are detectable in marine habitats adjacent to development. I sampled sea fans from the nearshore waters of Quintana Roo, Mexico, where the coastline is being increasingly developed to the south of Cancun. In Akumal, a developed resort town with a few thousand local residents, I sampled sea fans along a nearshore to offshore transect from 1 – 15 m depth. I also sampled corals along a 160 m transect within a lagoon suspected to be a significant source of sewage-polluted groundwater. For comparison, I sampled sea fans along a similar transect perpendicular to an undeveloped shoreline in Mahahual. Here, I show that gorgonians sampled near development in Akumal have enriched $\delta^{15}$N values, among the highest reported for corals (7.7 ‰). $\delta^{15}$N values of nearshore corals showed marked differences in N sources, despite previous reports that N concentrations are the same. High $\delta^{15}$N values were positively correlated with fecal enterococcus counts from
adjacent seawater, confirming that these enrichments are associated with sewage and not denitrification. I suggest that the data from Mahahual can be used as an isotopic baseline for monitoring the Meso-American barrier reef where increased development is underway.

**Introduction**

With burgeoning coastal populations, sewage pollution has become a serious threat to coastal marine systems where it disrupts ecosystem function through the inputs of nutrients, pathogens, and other contaminants (Camargo & Alonso, 2006). Sewage-derived nutrients, particularly nitrogen (N) and phosphate (P), alter the fundamental structure and function of marine systems by increasing primary productivity, exacerbating disease, and altering food web structure (Howarth et al., 2000). Generally oligotrophic, tropical marine systems are particularly disturbed by increased nutrient inputs from land. Indirectly, nutrients increase the proliferation of algae, epiphytes, suspension feeders, and bioeroders, which collectively smother, foul, and prevent recruitment of critical species like seagrasses and reef-building corals (Waycott et al., 2009). Directly, elevated nutrient concentrations disrupt the symbiotic association between corals and their zooxanthellae, and reduce fecundity. Taken together, the direct and indirect effects of nutrient pollution can induce a shift towards an algal-dominated benthos, which is characterized by a severe loss of biodiversity and ecosystem services (Knowlton, 2004, Littler et al., 2006). In addition to N and P, sewage effluents contain pathogens and contaminants that have been linked to coral disease and mortality (Patterson et al., 2002). These effects are particularly damaging to developing regions where public wellbeing and economic growth are dependant
Stable isotope analysis is a promising tool for monitoring changes in the contribution of human N sources to nearby ecosystems, especially where comprehensive water quality monitoring programs are not established. Specifically, the isotopic ratio of $^{15}$N to $^{14}$N is regarded as an effective and direct indicator of human nitrogen pollution. Generally, enriched isotope values (having relatively more $^{15}$N) arise from the accumulation and degradation of human and animal wastes. For example, nitrate in raw or partially treated sewage-contaminated groundwater can have $\delta^{15}$N values much greater than 10 ‰ (Katz & Griffin 2008), though fractionations are dependent on the method of sewage treatment. In contrast, N from upwelling has lower $\delta^{15}$N, averaging 4-7 ‰ (Leichter et al., 2007), while N fixed by diazotrophs is relatively depleted, averaging -1 to 0 ‰ (Karl et al., 2002). Thus, sewage derived N can be easily distinguished from natural marine sources, especially when sewage N comprises a major proportion of the total N pool.

While isotope ratios provide information as to the source of N within an ecosystem, local factors can complicate the interpretation and general applicability of conclusions. Several studies tracing N sources in highly polluted locations have illustrated significant relationships between $\delta^{15}$N and proximity to those sources (Marion et al., 2005, Risk & Erdmann, 2000). However, these examples may be exceptional as most developed coastlines are likely to have multiple sources of both natural and anthropogenic N. For example, Swart et al. found no compelling isotopic evidence to support the hypothesis that sewage derived N is impacting nearshore reefs in the Florida Keys. The Florida Keys have many point and non-point N sources as well as...
documented isotopic fractionations that alter the $\delta^{15}\text{N}$ of water column N (Corbett et al., 1999, Lapointe et al., 2004). Moreover, it has been shown that light is an important factor in influencing isotope values through metabolic fractionations sensitive to photosynthetic rate (Heikoop et al., 1998) and should be considered in isotope studies. While it has been stressed that attention should be given to characterizing local sources (upwelling, deposition, fixation, etc.) and identifying environmental transformations of N (denitrification, nitrification, etc.), there is also a need to corroborate isotopic signals of anthropogenic N sources with other environmental data.

The Mexican Riviera and Costa Maya are among the most rapidly developing regions in the world, situated along the coast of the State of Quintana Roo (Fig. 3.1). This region attracts more than 5 million tourists per year and provides a unique opportunity to examine the effects of coastal development and intensified sewage inputs to the coastal marine environment (Murray, 2007). Quintana Roo is characterized by a karst geology, which is so permeated with subterranean channels that there are few instances of surface waters in the region. Typically, groundwater conducted from inland discharges from benthic sinkholes into the coastal marine environment. En route to the sea, groundwater is easily contaminated by surface pollutants, particularly
from sewage and wastewater effluents from homes and resorts. Many local residences lack the means to properly dispose of sewage, while resorts are responsible for on-site wastewater treatment (ArandaCirerol et al., 2006, Pacheco et al., 2001). Much of the groundwater research in this region has been focused on the study of wells and sinkholes (“cenotes”) with concern for microbial and chemical threats to human health. Comparatively little attention has been given to the marine environment where polluted groundwater encounters sensitive seagrass and coral reef ecosystems. This is a critical area of study given the concentration of development adjacent to the sea, poor wastewater treatment practices, lack of marine water quality monitoring, and the potential consequences for the benthic marine environment upon which the tourism industry relies heavily.
Here I examine stable isotope ratios of gorgonian corals and enteric bacterial abundance to test the hypothesis that reefs adjacent to developed coastlines are polluted by sewage-contaminated groundwater. I asked: 1) do developed coastlines have higher $\delta^{15}$N nearshore and offshore as a result of greater sewage N inputs?, 2) can I identify sites <1 km apart that have greater sewage inputs alongshore?, 3) are high $\delta^{15}$N values from sewage inputs, or natural microbial denitrification? The answers to these questions are critical for developing cost-effective monitoring in rapidly developing coastal areas world-wide.

**Methods**

*Regional comparisons*

To test the hypothesis that development generates sewage pollution adjacent to developed coastlines, I sampled sea fan corals (*Gorgonia ventalina* L.) along a nearshore-offshore gradient from 2 to 15 m depth in Akumal (n = 90), which has a developed resort area and “colonia” (an inland community across the coastal highway), and Mahahual (n = 30), a relatively new development that reportedly maintains it’s own sewer and treatment system and has a much smaller colonia located approximately 200 km to the south. Both regions have the same karst geology, few instances of surface waters, and an abundance of benthic sinkholes or sandy depressions where groundwater percolates into the marine environment. Furthermore, the reef and coral community structure are similar between these regions. In both Akumal and Mahahual the 15 m reefs sampled are ~1 km from shore, however, corals are more abundant and cover is slightly higher in Mahahual (15 %) vs. Akumal (13.8 %; Jordán, et al. in preparation).
In Akumal there is a coastal lagoon formed by a geological fracture named “Yalku” from which a substantial groundwater source has eroded the surface limestone, and terminates at a well-formed reef crest where it meets the sea. Beginning in the middle of the lagoon where sea fans are present (Yalku Mid) and ending offshore at Yalku Deep, nine sites were sampled approximately every 1.5 m depth (Fig 3.1a). In Mahahual, three sites were sampled from near to offshore; Mahahual Backreef, Mahahual 10m, and Mahahual 15 m (Fig 3.1b). Sampling began with the first occurrence of sea fans in shallow waters, and ended approximately 1 km from the shoreline at the 15 m sites. All samples were collected in August of 2008.

Identifying point-sources of N pollution

In 2008, I sampled sea fans from the north-facing wall of Yalku Lagoon, approximately every 20 m from the first specimen to the reef crest to determine fine-scale variation in isotope values. All samples were collected from < 2m depth. In the summers of 2004 - 2008, I sampled sea fans from the shallow forereef parallel to shore to determine areas of isotopic enrichment. In Akumal, 7 sites were sampled at various depths; Yalku Lagoon, Xa’ak, La Escuela, Las Redes, Half Moon Bay, Akumal, Bahia Principe, and South Akumal (Fig. 3.1a).

At each site approximately five 2 cm$^2$ fragments of sea fan were sampled from the colony edge using SCUBA or snorkel. This area likely represents the previous year of growth (Cary, 1914). All samples were immediately taken to shore where they were air-dried and stored prior to shipping to Cornell University.
Stable isotope analyses

Each sample was homogenized by grinding in a SPEX Certiprep cryogrinder using liquid nitrogen. The resulting powder, consisting of the skeletal axis, coenenchyme, polyps, and zooxanthellae was weighed into 4 x 6 mm tin capsules, which were combusted in a Carlo-Erba elemental analyzer and analyzed by a Finnegan-MAT Delta Plus isotope ratio mass spectrometer. Reported $\delta^{15}$N and $\delta^{13}$C values are relative to atmospheric N$_2$ and Vienna Pee Dee Belemnite (VPDB), respectively. Precision for $\delta^{15}$N and $\delta^{13}$C was quantified by two in-house standards [bcbg (0.08 and 0.06 ‰, respectively) and methionine (0.09 and 0.1 ‰, respectively)] calibrated against IAEA international standards. Additionally, I used an in-house homogenized sea fan standard, which had a precision for $\delta^{15}$N and $\delta^{13}$C of 0.05 and 0.25 ‰, respectively.

Enterococcus Assays

To determine if N isotope values are correlated with sewage pollution along the coast, I quantified the prevalence of fecal enterococcus bacteria in surface waters adjacent to sea fans sampled at 10 sites, 7 of which were nearshore in the protected backreef (Yalku Mid, Half Moon Bay, South Akumal, and four sites from the lagoon in Puerto Morelos) and three sites were just behind the reef crest (Akumal Bay, Bahia Principe, and Yalku Crest). Three 100 mL seawater samples were collected from each site in sterile Whirl-paks and transported to the lab on ice. The samples were allowed to settle, and an 80 mL aliquot was vacuum filtered through a 47 mm sterile, grid-marked membrane filter (0.45 um pore size). The filter was removed with sterile forceps and placed into a 9 x 50 mm petri dish containing ~5 mL m-Enterococcus agar (Difco; EPA Method 1600). This media selectively cultures bacteria from the fecal
streptococci subgroup of enterococci including Enterococcus faecalis, E. faecium, E. gallinarum, and E. avium, which appear red on the membrane surface. The plates were incubated at 37 C for at least 48 hours, or until colonies were observed and countable. Individual colonies were counted using a magnifying glass under fluorescent light. Each set of incubations was conducted with replicate negative controls (sterile deionized water) and positive controls using a pure culture of E. faecalis. Data reported are colony-forming units (CFUs) per 100 mL.

**Statistical Analysis**

Prior to analysis, all data were screened for normality and homoscedasticity using Shapiro-Wilk and Levene’s tests, respectively. I used analysis of covariance (ANCOVA) on log-transformed data to test the null hypotheses that sea fan δ¹⁵N, δ¹³C, and %N, %C, and C/N; 1) are not different between regions, 2) do not vary with depth, and 3) change over depth at the same rate between regions (depth*region). Within each region, I tested the null hypothesis that δ¹⁵N and δ¹³C of sea fans sampled at individual sites (reefs or hard bottom communities) are similar. Significant differences between sites were determined using ANOVA followed by pairwise Student’s t-tests.

A similar approach was taken for the bacterial data. For within region site comparisons, the bacterial counts were transformed to meet the assumptions of normality and homoscedasticity for parametric analyses using a Box-Cox y transformation. Differences among sites within the region were determined using ANOVA. Specific differences between sites were determined using post-hoc pairwise Student’s t-tests. The relationship between δ¹⁵N and Enterococcus counts was
determined using linear regression. All statistical analyses were conducted in JMP 7.0 (SAS Institute, Cary, NC).

**Results**

*Isotopes and elemental ratios*

Both %C and %N of *G. ventalina* varied with depth in both developed (Akumal) and undeveloped (Mahahual) regions. Overall, %C was highest nearshore and decreased with depth, ranging from 22.4 – 26.4% in Akumal, and 21.1 – 25.6% in Mahahual. Similar declines with depth were seen in %N, which ranged from 2.5 – 3.9% in Akumal, and 2.9 – 4.0% in Mahahual. There was no difference between Akumal and Mahahual, nor in the rate of change over depth between regions for either % element (Table 3.1).
Table 3.1. Means ± standard deviation for elemental and isotopic data for all sites, including the years they were sampled.

<table>
<thead>
<tr>
<th>REGION</th>
<th>SITE</th>
<th>YEAR</th>
<th>n</th>
<th>DEPTH (m)</th>
<th>%N</th>
<th>$\delta^{15}N$</th>
<th>%C</th>
<th>$\delta^{13}C$</th>
<th>C:N</th>
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<td>AKUMAL</td>
<td>AKUMAL BAY</td>
<td>'06, '08</td>
<td>10</td>
<td>2</td>
<td>4.4 ± 0.2</td>
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<tr>
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<td>'08</td>
<td>5</td>
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<td>4.5 ± 0.3</td>
<td>6.4 ± 0.1</td>
<td>26.4 ± 0.7</td>
<td>-11.4 ± 0.2</td>
<td>5.8 ± 0.2</td>
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<td>6</td>
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<td>4.5 ± 0.1</td>
<td>25.5 ± 0.6</td>
<td>-11.7 ± 0.3</td>
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<td>-11.4 ± 0.2</td>
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<td>3</td>
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<td>6.5 ± 0.2</td>
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<td>28.7 ± 0.4</td>
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<td>CHANNEL</td>
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<td>26.3 ± 1.0</td>
<td>-10.9 ± 0.4</td>
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<td>15</td>
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<td>27.4 ± 0.6</td>
<td>-12.6 ± 0.2</td>
<td>5.6 ± 0.1</td>
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Figure 3.2. Nearshore to offshore trends in mean C/N (A) and $\delta^{15}$N (B) in Akumal (black) and Mahahual (grey) of *G. ventalina* collected in 2008. Bars represent standard error. Lines represent significant power (log-log) regressions. The star (★) represents the mean $\delta^{15}$N of nitrate in Yalku Lagoon as reported by Mutchler *et al.* (2007).
Overall, $\delta^{15}$N and $\delta^{13}$C of *G. ventalina* ranged from 0.9 to 7.7 and -17.6 to -6.6 ‰, respectively across the depth gradient. Sea fans from shallow sites nearshore were consistently enriched in both isotopes relative to deeper, offshore sites. In Akumal, sea fan $\delta^{15}$N ranged from 7.7 ‰ closest to the groundwater source in Yalku Lagoon (1 m) to 2.1 ‰ at Yalku Deep (15 m) offshore (Fig. 3.2b). Samples from Mahahual showed a similar trend, though a smaller range, from 3.6 to 1.5 ‰, near- to offshore (Fig. 3.2b).

To test for the effect of depth, region, and depth*region on %C, %N, C/N, $\delta^{15}$N, and $\delta^{13}$C I used analysis of covariance on log-transformed factors (ANCOVA; Table 3.2). There was a significant effect of depth on all factors. $\delta^{15}$N, %C, and %N all decreased with increasing depth, while $\delta^{13}$C and C/N increased over the depth gradient. There were significant differences between regions, with Akumal having higher C/N, $\delta^{15}$N, and lower $\delta^{13}$C than Mahahual. However, there were no differences detected in either %N or %C between the regions. Only $\delta^{13}$C produced a significant depth*region interaction, which was driven by very low values in samples from the shallow waters of Yalku Lagoon which were relatively depleted by 2.2‰ compared to samples from the Mahahual backreef, thus increasing the slope of the regression.

*Fine-scale isotope and elemental trends*

$\delta^{15}$N and $\delta^{13}$C varied among sea fans sampled along a 160 m transect in Yalku lagoon. $\delta^{15}$N was highest in sea fans closest to the groundwater source (7.7‰), though there was another spike in $\delta^{15}$N at 140 m, followed by a gradual decline towards the reef crest (6.9‰). However, this slope of this decline was not different from zero ($R^2 = 0.33$, $p = 0.10$; Fig. 3.3a). There was a significant increase in $\delta^{13}$C along the same transect from -17.6 to -13.8 ‰ ($R^2 = 0.67$, $p = 0.006$; Fig. 3.3a). C/N ratios increased
Table 3.2. Results of ANCOVA with log-transformed factors. Bold values indicate statistical significance at alpha = 0.05. The last column (AK vs. MH) represents the relative differences between factor means between regions using post-hoc Student’s t-test. nd = no statistical difference.

<table>
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<th>FACTOR</th>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
<th>AK vs. MH</th>
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<tr>
<td></td>
<td>REGION*DEPTH</td>
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<td>0.531</td>
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<tr>
<td>%N</td>
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<td>0.094</td>
<td>nd</td>
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<tr>
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<td><strong>0.0019</strong></td>
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along the transect, though this trend was not statistically significant ($R^2 = 0.38, p = 0.07$; Fig. 3.3b). Variation in C/N was driven by changes in %N ($R^2 = 0.67, p = 0.007$), not %C ($R^2 = 0.00, p = 0.99$).

Multi-year comparisons between sites in Akumal

There were significant differences between sites parallel to shore in Akumal with respect to δ¹³C (ANOVA; $n = 77, F_7 = 17.04, p < 0.0001$; Fig. 3.4a), though this difference was driven by relatively low δ¹³C values in Yalku Lagoon, the other sites were similar. There was greater variation in δ¹⁵N (ANOVA; $n = 77, F_7 = 50.33, p < 0.0001$; Fig. 3.4b). Yalku lagoon was significantly enriched in ¹⁵N relative to other sites,
Figure 3.3. Trends in stable isotope values (A) and C/N (B) from *G. ventalina* sampled over a 160m transect in Yalku Lagoon away from a groundwater source in 2008. Points represent individual samples. There was a significant correlation between δ¹³C and distance, but not in the other parameters.
Figure 3.4. Mean $\delta^{13}$C (A) and $\delta^{15}$N (B) among G. ventalina sampled at sites parallel to shore in Akumal averaged for all years sampled between 2004-08. Bars represent standard error. Groups not sharing letters (or asterisk) were significantly different.
followed by Akumal Bay and Bahia Principe. Las Redes and Xaak had the lowest δ^{15}N values. There was little year-to-year variation in isotope ratios, for example, the 10 m site outside of Yalku lagoon varied by 0.7 ‰ over the years sampled.

Figure 3.5. Mean Enterococci colonies cultured from triplicate 100 mL water samples along a fresh to saltwater gradient within Yalku Lagoon. Note that the mean CFUs from the lagoon source are nearly 2 times higher than the USEPA limit for recreational waters. Bars represent standard error.

*Enterococcus*

Groundwater originating in Yalku lagoon was a source of *Enterococci* (mean ± SE = 59 ± 3.5 colony forming units (CFU)/100mL), though the presence of culturable bacteria declined with distance from the source, with less than 1 CFU/100mL at the reef crest (Fig. 3.5). Throughout Akumal, there were differences between sites with
Figure 3.6. Mean *Enterococci* colonies cultured from triplicate 100 mL water samples parallel to shore, from North to South, in Akumal. Columns with different letters signify significant differences between means. Bars represent standard error.

respect to the prevalence of *Enterococci* in surface waters (ANOVA, \( n = 15, F_4 = 10.7, p = 0.0012 \); Fig. 3.6). Water samples from Yalku lagoon and Half Moon Bay yielded significantly more bacterial colonies than Akumal Bay, South Akumal, or Bahia Principe (Student’s t-test, all \( p < 0.05 \)).

In Mahahual, there were differences between sites in *Enterococcus* prevalence (ANOVA, \( n = 18, F_5 = 25.5, p < 0.0001 \); Fig. 3.7). This was driven by high CFUs in samples adjacent to the Luna de Plata site (28 ± 5.3 CFU/100mL), which is just beyond the end of the town’s sewer line. This site was unique among the other sites sampled from the sewered areas in town, and from more remote sites (Student’s t-test,
Figure 3.7. Mean *Enterococci* cultured per 100mL water sampled parallel to shore, from North to South, in Mahahual. The column with an asterisk signifies a significant difference in average colony counts. Bars represent standard error. The dashed line represents the separation of sites sampled inside and outside the municipal septic line.

all $p < 0.05$). Bacterial counts were low in the town center, which typically has the highest density of swimmers and bathers (pers. observation).

There was a significant positive correlation between $\delta^{15}$N and *Enterococcus* counts from sites sampled from low energy lagoon areas ($n = 7$, $R^2 = 0.66$, $p = 0.025$; Fig. 3.8). There was no correlation found among high-energy reef crest sites, although these sites were still relatively enriched in $^{15}$N.
Discussion

Our results show clear differences in the predominant N sources assimilated by sea fan corals along the coast of Quintana Roo, consistent with the known isotopic influence of sewage derived N. Elevated isotope ratios were observed along the developed coastline and were correlated with the abundance of enteric bacteria, which supports our hypothesis that these inputs are from sewage and not microbial denitrification of natural groundwater N sources. In line with our hypothesis, $\delta^{15}$N of sea fans from Akumal revealed significant enrichment, relative to Mahahual (Fig 3.2a). The observed difference between nearshore sea fans was $\sim 3.5 \%e$ which is equivalent to the average difference observed between trophic levels within a food web (Deniro & Epstein, 1981, Minagawa & Wada, 1984).
Based on previous work on the diet of gorgonians, this enrichment is not likely due to predation upon a naturally enriched N source. Gorgonians primarily consume dissolved and particulate organic matter (Lasker, 1981) in such low proportion that heterotrophy alone is not sufficient to meet N demands for growth and reproduction (Ribes et al., 1998). Instead, these corals likely assimilate inorganic N via direct uptake by their symbiotic zooxantellae, thus, the differences in $\delta^{15}$N can be attributed to an inorganic N source. Mahahual’s low isotope ratios nearshore (< 4 ‰) are similar to values reported for peat N (Heaton, 1986, Hoering, 1955, Lapointe et al., 2004), which originates from mangrove swamps and leaches into the marine environment becoming significant source of N. Within Akumal, three nearshore sites had $\delta^{15}$N greater than 6 ‰. I hypothesized that sewage pollution in Akumal was the driver of this isotopic enrichment, given the relatively low $\delta^{15}$N of nearshore corals in Mahahual, adjacent to nearly pristine areas. Thus, $\delta^{15}$N values of sea fans along the depth gradient in Mahahual are a useful baseline from a pristine environment, which I can use to compare impacted areas along the coast.

Within Akumal, I chose Yalku Lagoon as a target for increased spatial sampling to characterize the relationship between $\delta^{15}$N, distance, and depth, due to the enrichments seen there (up to 7.7 ‰). Yalku Lagoon is a known source of N to the coastal marine environment with NO$_3^-$ concentrations greater than 25 $\mu$M (Mutchler et al., 2007). Mutchler et al. (2007) conducted isotope analyses of NO$_3^-$ in Yalku Lagoon, reporting $\delta^{15}$N values of 7.6 ‰. That the $\delta^{15}$N of G. ventalina nearest to the groundwater source were as high as 7.7 ‰ suggests that sea fans are assimilating NO$_3^-$ as an N source, with no fractionation. In comparison to Mahahual, the relative $^{15}$N enrichment among sea fans from Akumal persisted along the depth gradient and was still higher by 0.5 ‰.
at 15m depth illustrating that sources of N from land are impacting reefs nearly 1 km from the shoreline (Fig. 3.2b), and that simple dilution by seawater is not effective at mitigating the impact of N pollution. Reports of enriched $\delta^{15}$N along this developed coastline suggest that sewage contamination is widespread. Ward-Paige et al. (2005) recorded $\delta^{15}$N values >7 ‰ off of Xel-Ha, just 7 km to the south of our southernmost site in Akumal. Using Mahahual as a pristine baseline, isotope ratios >4 ‰ may be an indication of sewage pollution in similar karst-based tropical marine environments, though this threshold should not be extended to other areas of the Caribbean with different geologies, topographies, or land-use.

The *bulk* C isotope ratios of gorgonians were not as sensitive to sewage pollution as $\delta^{15}$N. $\delta^{13}$C values were depleted in Yalku lagoon relative to other sampling locations. This suggests that corals close to the groundwater source in Yalku Lagoon are consuming dissolved and particulate organic matter from sewage, which can be -23 to -26 ‰ (Ramirez-Alvarez *et al.*, 2007, Rogers, 2003). $\delta^{13}$C also changed rapidly along a 160 m transect in Yalku Lagoon, becoming progressively enriched, by ~4.2 ‰ (Fig. 3.3a), relative to $\delta^{15}$N which decreased by 0.8 ‰ over the same distance. That the range in $\delta^{13}$C was much greater than that of $\delta^{15}$N suggests that marine C sources quickly dilute any C isotopic signal from sewage, which is not the case for N. This may be why $\delta^{13}$C was not significantly different among other sites sampled parallel to shore (Fig. 3.4a) that were significantly different with respect to $\delta^{15}$N (Fig. 3.4b). I did not remove lipid or carbonates from these samples, which makes interpretation of the dietary source of C difficult.

Fine scale patterns in C and N isotope and elemental ratios of sea fans within Yalku Lagoon are clearly driven by a sewage source (Fig. 3.4), not light mediated isotope
effects (Heikoop et al., 1998), or denitrification as proposed by Mutchler et al. (2007). Light-dependent isotope fractionation would manifest as progressively declining isotope ratios with depth, as I have reported. However, sea fan $\delta^{15}$N from within Yalku Lagoon declined with distance from the source, even though they were collected from the same depth (Fig. 3.4a). Furthermore, denitrification within the sediments would enrich $\delta^{15}$N with distance, which I did not observe. Perhaps the spike in $^{15}$N mid-transect is a result of denitrification in the sandy area in the center of the lagoon, or it could be evidence of a point-source wastewater input from residences build upon the lagoon wall. Although C/N did not vary significantly along the wall, there was a general trend of increasing C/N away from the groundwater source. This variability was driven entirely by the variation in %N, which was highest near the source, and not %C. A recent study by Alamaru et al. (2009) demonstrated that C/N, as an indicator of stored lipids, declined over a depth gradient in the hexacoral *Stylophora pistillata*. However, a similar trend was not seen in *Favia fragus*, which the authors ascribed to consistent heterotrophy at all depths. Their study concluded that high C/N in shallow waters is a reflection of autotrophy, and low C/N at depth an indication of heterotrophy. The data I present here are completely contrary to this finding, as the lowest C/N was consistently found closest to shore and increased with depth. Given the results of Alamaru et al., that sea fan C/N varied with depth confirms that *G. ventalina* is relatively autotrophic, similar to *S. pistillata*. However, I did not set out to specifically quantify the C/N of lipids, nor did I remove lipids prior to isotope analyses, thus I do not claim that the observed trends in C/N over the nearshore-offshore gradients in this study are due to lipid content. Since variation in C/N was correlated to %N, not %C suggests that N does not limit nearshore corals as they are growing in a high N environment. Furthermore, these data suggest that N is not limiting to corals in Akumal and Mahahual, thus the differences I see in $\delta^{15}$N between
these regions are not due to the fractionating effects of N-limitation but due to differences in the source N.

Gorgonians are important targets for isotope analyses, as they sequester N within their tissues and skeletal elements, thus preserving a weighted average of the predominant N sources over time, and therefore may be better suited for assessing long-term sewage stress than measuring water column N concentrations or isotope ratios of ephemeral benthic organisms like macroalgae (Risk, 2009, Risk et al., 2009). For example, our data show that δ₁⁵N from corals in Akumal Bay are 3.5 ‰ enriched relative to Xaak. This suggests that there is relatively less sewage N present in Xaak, despite the similar NO₃⁻ concentrations reported by Mutchler et al. who, using N to P ratios and NO₃⁻ concentrations in seawater, concluded that Akumal’s nearshore marine habitats are not N-limited, which is typical for tropical lagoon systems (Corredor et al., 1999). Despite differences in local development (between Akumal Bay and Xaak) they found no significant differences in NO₃⁻ concentrations, and they concluded that there was no difference in the N sources between Akumal Bay and Xaak. Our sea fan δ¹⁵N data clearly show distinct differences between sources of N reaching bays and reefs throughout Akumal (Fig. 3.4b). Sites sampled in Akumal Bay and Bahia Principe, both heavily developed with hotels and resorts, were the most enriched sites next to Yalku Lagoon. Mutchler et al. claimed an inability to detect anthropogenic inputs at bay sites due to mixing, the small scale of sources, ephemeral inputs, and N removal during transport. One possibility for the lack of congruence between our study and that of Mutchler et al. is that macroalgae, having higher rates of N turnover, are better suited as a short term integrator (days to weeks) of ¹⁵N than sea fans which integrate N over seasonal to annual timescales (Gartner et al., 2002, Risk, 2009, Risk et al., 2009). Thus, macroalgae may capture short-term spikes in sewage derived N
subsequent to episodic rainfall or peak tourist visitation, and conversely, show rapid returns to marine $^{15}$N signals when sewage inputs wane (Lapointe et al., 2004).

With N isotope studies such as ours, it is critical to recognize that natural processes can produce enriched isotope ratios within the expected range for sewage-derived N. Denitrification is a logical alternative explanation for high $\delta^{15}$N values in groundwater. Therefore, it was important to corroborate the stable isotope data with another metric to support our hypothesis that observed $^{15}$N enrichments were due to enriched sewage derived N as opposed to a residual N pool that has reduced $^{14}$N due to denitrifying microbes. The results of the fecal enterococcus assay confirmed that the groundwater source flowing into Yalku lagoon is contaminated by sewage. Surface waters collected from the origin of the lagoon had the highest *Enterococcus* counts of this study (~$59$ CFU/100mL). The presence of elevated fecal *enterococci* well above the USEPA standard for recreational waters ($35$ CFU/100mL) is disconcerting, as this lagoon is a popular destination for tourists. It is possible that enteric bacteria are originating from bathers, yet high colony counts were recorded from samples from the nearby cenote where there are no swimmers, confirming that the bacteria are present in groundwater. Furthermore, Akumal Bay has the highest concentrations of bathers and swimmers, yet had comparatively low culturable *enterococci*, suggesting that contamination from swimmers is minimal. The inland *colonia* and residences are probable sources for this contamination, as nearly 20% of habitations in the region are not connected to any sewage system.

In contrast to Akumal, Mahahual maintains a sewage treatment center that connects the first kilometer of residences and businesses to a sewage treatment facility (Cpt. Lucio, pers. comm; Fig. 3.1b). *Enterococci* were low in Mahahual’s town center,
despite the high abundance of swimmers in the water, and all sewered sites sampled were indistinguishable from remote areas (Lighthouse and Casa de Arturo). Interestingly, there were significantly more bacteria cultured from the Luna de Plata site, which is situated just beyond the end of the septic line. Because the Luna de Plata is a hotel and restaurant, elevated Enterococci here could be directly coming from its wastewater, or there could be leakage from the sewage pipe. Still, Mahahual had significantly less bacterial counts than Akumal, and no instances above the USEPA standard. Continued monitoring, especially around the Luna de Plata site, is crucial for detecting major increases in sewage contamination. I were unable to determine exactly how the sewage was being treated in Mahahual, but our bacterial evidence suggests that the septic line is effective at maintaining low levels of bacterial contamination in the nearshore waters adjacent to the most densely populated areas, which I assume is simply by preventing leeching into the ground. Given the interest in developing Mahahual, these data should be used in support of continued investment in sewage treatment infrastructure.

The correlation between $\delta^{15}$N and Enterococcus shows that in calm waters, high sea fan $\delta^{15}$N was associated with high concentrations of bacteria in surface waters. The low abundance of bacteria cultured from reef crest waters could be due to progressive dilution of contaminated groundwater with seawater, bacterial mortality, mixing, or settlement to sediments. Future work should focus on quantifying the presence of enteric bacteria from sediments as a more integrated measure of contamination (Fries et al., 2006). In high-energy sites, I see that enteric bacteria are not a reliable indicator alone, as they attenuate rapidly over space, while sewage-derived N travels further and is detectable offshore, perhaps due to saturation of N in this environment. Previous studies have shown that sheltered lagoons along the coast have water is retained for
several days (Coronado et al., 2007). This may allow enteric bacteria to accumulate, increasing the probability of sampling culturable bacteria in the water.

Conclusions

Coastal populations in the Caribbean are expected to grow 40% by 2050 (UN, 1999). As a result, sewage pollution will worsen and accelerate the degradation of sensitive marine habitats. Thus, the challenge of cost-effective environmental monitoring will be met by studies measuring the isotopic ratios of benthic marine organisms as recorders of increasing pollution. This study contributes further evidence that isotope ratios from gorgonian corals are a particularly valuable tool for detecting sewage pollution in the absence of water quality monitoring, and are more useful for detecting pollution among sites with similar N concentrations. Through time-series sampling and relative comparisons between pristine and impacted environments, gorgonian corals can effectively capture perturbations in the ambient N pool due to human development. I have shown that coral reefs along the developed Mexican Caribbean are receiving an enriched N source, up to 1 km from shore, and that these sources correspond with elevated levels of enteric bacteria. Furthermore, elevated $\delta^{15}$N was correlated with high bacteria counts, but only in nearshore lagoon sites where bacterial dilution and mortality are likely lowest. Therefore, $\delta^{15}$N is sensitive to sewage pollution, even in areas where fecal bacteria cannot be detected by conventional means.

The $\delta^{15}$N data I present here are key to our understanding of coral decline in the Meso-American barrier reef. Since 2006, comprehensive assessments of coral diseases among high and low development areas within the Mexican Yucatan peninsula have
shown significant differences in the prevalence of Yellow Band Syndrome (YBS) among the *Montastrea annularis* species complex, now the dominant reef-building corals in the Caribbean. YBS is nearly 2 times more prevalent in Akumal than in Mahahual, and these stable isotope data indicate that there are greater sewage inputs to reefs along developed coastlines (Jordán *et al.*, in preparation). Since *in situ* experimentation has shown the direct association between nutrients and YBS severity (Bruno *et al.*, 2003) and YBS is more prevalent in Akumal, it is likely the mortality of these corals due to disease are a result of sewage pollution. Moreover, remediation of sewage pollution should ameliorate the decline of these reefs from disease, and increase their resilience to global climate change. Mahahual offers a unique setting for documenting the impacts of development on nearshore marine habitats as the government has targeted this town for accelerated development for tourism. Long-term monitoring of both the coral community and the isotope biogeochemistry of gorgonians in Mahahual will allow us to document the impact of development as it occurs as I now have a baseline from which I will compare future data.
REFERENCES


CHAPTER 4

CARRIBEAN OCTOCORALS RECORD CHANGING CARBON AND NITROGEN SOURCES FROM 1862-2005

Abstract

During the last century, the global biogeochemical cycles of carbon (C) and nitrogen (N) have been drastically altered by human activities. A century of land-clearing and biomass burning, followed by fossil fuel combustion have increased the concentration of atmospheric CO$_2$ by approximately 20%, and since the mid-1900s, the use of agricultural fertilizers has been the primary driver of an approximate 90% increase in bioavailable N. Geochemical records obtained through stable isotope analysis of terrestrial and marine biota effectively illustrate rising anthropogenic C inputs. However, there are fewer records of anthropogenic N, despite the enormous magnitude of change and the known negative effects of N on ecosystem health. I used stable isotope values from independent octocorals (gorgonians) sampled across the Western Atlantic over the last 143 years to document human perturbations of the marine C and N pools. Here, I demonstrate that in sea plumes δ$^{13}$C values and in both sea plumes and sea fans δ$^{15}$N values declined significantly from 1862 to 2005. Sea plume δ$^{13}$C values were negatively correlated with increasing atmospheric CO$_2$ concentrations and corroborate known rates of change resulting from global fossil fuel combustion, known as the Suess effect. I suggest that widespread input of agricultural fertilizers to near-shore coastal waters is the dominant driver for the decreasing δ$^{15}$N trend. Given the interest in using δ$^{15}$N as an indicator for N pollution in aquatic systems, I highlight the risk of underestimating contributions of pollutants as a result
of source mixing, as demonstrated by a simple isotope-mixing model. I conclude that signals of major human-induced perturbations of the C and N pools are detectable in specimens collected over wide geographic scales, and that archived materials are invaluable for establishing baselines against which I can assess environmental change.

**Introduction**

Geochemical records of anthropogenic change have received much attention in recent decades for their use in revealing our impacts on the biosphere. The work of Francey, Freyer, Suess, and others have utilized both radio- and stable carbon isotope analyses of tree rings as records for illustrating the rise in anthropogenic CO$_2$ as a result of fossil fuel combustion and biomass burning (Francey *et al.*, 1999, Freyer & Belacy, 1983, Suess, 1955). Their pioneering works provided a solid link between human development and global change. Since that time, declining trends in $\delta^{13}$C have been observed in a variety of taxa (Bump *et al.*, 2007) including those in the marine environment (Druffel, 1997, Druffel & Benavides, 1986). Many studies have effectively demonstrated that sponges and corals offer similar records for global change, mirroring their terrestrial counterparts. Surprisingly, relatively less attention has been given to the perturbation of the global nitrogen (N) cycle, despite the implications for ecosystem degradation, biodiversity, and long-lived greenhouse gasses. Given the fidelity of anthropogenic C signals across the land-sea boundary from an approximate 20% increase in global CO$_2$, I expect that similar records for N perturbations are widespread, given the nearly 90% increase in reactive N in our biosphere (Galloway *et al.*, 2004, NRC, 2000).
While many have documented the direct and indirect impacts of N pollution from organisms to ecosystems, the challenge of determining the provenance of N from a myriad of point and non-point sources persists. The widespread use of synthetic agricultural fertilizers has been a major driver of eutrophication in coastal marine waters (Howarth et al., 2005). These effects are especially pronounced in the tropics where the community structure of generally oligotrophic environments is rapidly altered upon release from N-limitation. This is especially true for coral reefs. Sensitive to an overabundance of N, coral reefs are known to shift to an alternate ecosystem state dominated by algae and suspension feeders (Knowlton, 2004, Lapointe, 1997). Phase-shifts reduce biodiversity and habitat availability, which ultimately depletes the services these ecosystems provide. However, little is known about the major sources of N added to the oceans since industrialization. Reconstructing historical trends and baselines for the dominant N sources is essential for identifying and devising effective conservation management strategies so that direct and indirect stressors resulting from anthropogenic N can be alleviated, thus increasing the health and resilience of coral reefs (Risk, 2009).
Figure 4.1. Fertilizer consumption from 1860 to 2005 in the USA (solid line) and the Caribbean (dashed line). Data acquired from the Food and Agriculture Organization of the United Nations (FAO-STAT)

Since the 1960s, fertilizer use in the Caribbean has increased approximately six-fold, a clear signal of the agricultural ‘Green Revolution’, concomitant with rapid coastal development (FAO-STAT; Fig. 4.1). Unfortunately, there is a considerable paucity of data from this critical time of change pertaining to how the increasing application of synthetic fertilizers has impacted reefs throughout the Western Atlantic. Biogeochemical records from marine invertebrates have elucidated the profound effects of human activities on the biosphere. Long-lived organisms like corals and sponges provide excellent records for environmental change because they accumulate growth, sometimes in bands of recalcitrant material, which integrate environmental conditions from the time of formation. Stony (scleractinian) corals have been particularly useful for reconstructing past sea surface temperatures (Beck et al., 1992), ocean pH (Honisch et al., 2004), anthropogenic CO$_2$ emissions (Quinn et al., 1998),
and more recently, nitrogen pollution from sewage and synthetic fertilizers (Marion et al., 2005, Ward-Paige et al., 2005). The latter two records have been identified through analysis of the stable isotope ratios of C and N, respectively, which are fractionated by human activities. In short, the extraction and combustion of fossil fuels derived from ancient plants contributes $^{12}\text{CO}_2$ to the atmosphere, lowering the $\delta^{13}\text{C}$ of the gaseous CO$_2$ pool (Craig, 1954, Suess, 1955). Human alteration of the isotopic ratio of N are varied, with trophic enrichment and microbial processing creating high $\delta^{15}\text{N}$ in human sewage effluents (Savage, 2005), while the synthesis of nitrogenous fertilizers and atmospheric deposition yields $^{15}\text{N}$-depleted compounds that lower the $\delta^{15}\text{N}$ of connected ecosystems (Barile & LaPointe, 2005, Marion et al., 2006). Recent work has shown that corals are accurate recorders of fertilizer N. Marion et al. (2005) showed that increased fertilizer use in Indonesia was associated with a drop in skeletal $\delta^{15}\text{N}$ of nearshore corals by $\sim$10 $\%_\circ$ over 25 years. Furthermore, a retrospective analysis of corals from the ENCORE experiments conducted on One Tree Island, Australia revealed a significant drop in $\delta^{15}\text{N}$, from $\sim$3.5 $\%_\circ$ to less than 1.0 $\%_\circ$ from both corals and their symbionts in experimental patch reefs treated with nitrogenous fertilizers (Hoegh-Guldberg et al., 2004).

Museum collections offer a unique source of material for retrospective investigations of global change. Several studies on the effects of preservation have shown that $\delta^{15}\text{N}$ is relatively unchanged by time or preservatives in animals, though protein and lipid-rich tissues are more sensitive to isotopic change (Kaehler & Pakhomov, 2001, Sweeting et al., 2004). Geochemical analyses of stony coral skeletons may be confounded by endolithic organisms, which can alter the stable isotopic composition of the skeletal organic matrix, a compartment that comprises less than 0.02% of the skeletal mass (Marion et al., 2006, Nothdurft & Webb, 2009). Unlike stony corals,
gorgonians accrete protein-based skeletons that are highly resistant to diagenesis. Both dried and fossilized gorgonians retain their protein and stable isotopic composition (Sherwood et al., 2006). In light of the dramatic increases anthropogenic C and N since the mid-1900s, I determined the bulk $\delta^{15}$N and $\delta^{13}$C of museum-held gorgonian corals, collected over 143 years (1862 – 2005), to assess the change in coastal C and N sources as a result of increasing human perturbation. Here I present data from individual museum specimens of Caribbean gorgonians (Class: Anthozoa, Family: Gorgoniidae) sampled over a century of industrialization to test the hypothesis that global change within the coastal oceanic C and N pools are detectable across large spatial and temporal scales.

Methods

Symbiotic (zooxanthellate) gorgonians inhabit near-shore hard bottomed habitats to ~50 m depth, areas highly sensitive to coastal development. In this study, I sampled 95 specimens representing five species: Gorgonia flabellum ($n = 6$), G. ventalina ($n = 21$), Pseudopterogorgia acerosa ($n = 30$), P. americana ($n = 27$), P. bipinnata ($n = 6$), and P. spp. ($n = 5$). These species maintain an obligate symbiosis with a photosynthetic dinoflagellate (Symbiodinium). I chose these species for our study based on several lines of evidence that led to our assumption that these corals are primarily autotrophic and acquire minimal contributions from heterotrophy. First, while a poor predictor of nutritional mode, large polyp size is thought to correlate with predation upon larger food particles (Yamamuro et al., 1995). Sea plumes and sea fans possess small, nematocyst-poor polyps, which contract at night when plankton abundance is highest on reefs (Lasker, 1981). Second, gut-content analyses have shown that gorgonians feed on particulate organic matter and zooplankton in the 100 –
700 um size range. However, these prey comprised 0.4 and 17% of the annual C and N demand for growth and reproduction, respectively, while the rate of prey capture is among the lowest recorded in corals (Ribes et al., 1998). Ribes et al. further observed a loss of N through the regular expulsion of zooxanthellae, and hypothesized that uptake of inorganic N through the symbionts must be important for meeting N demands. Indeed, isotope values from sea fans from a nearshore lagoon in Mexico have isotope ratios that are nearly identical to the $\delta^{15}N$ of nitrate in the water column, which suggests that inorganic N is an important N source for gorgonians and is incorporated with little fractionation (Baker, unpublished, (Mutchler et al., 2007). Thus, the $\delta^{13}C$ and $\delta^{15}N$ of these species tissues are an accurate reflection of the C and N isotopic baseline of the ecosystem.

Dry museum specimens were obtained from the Smithsonian National Museum of Natural History Invertebrate Zoology Collection (Washington, DC), the Harvard University Museum of Comparative Zoology (Cambridge, MA) and the Yale Peabody Museum (New Haven, CT). These samples originated from 4 regions of the Caribbean; the Greater Antilles (Cuba, Dominican Republic, Jamaica, Puerto Rico, Turks & Caicos, Cayman Islands), the Lesser Antilles (Dominica, Guadalupe, the Netherlands Antilles, USVI), the North Atlantic (Bahamas, Bermuda, Florida), and the Yucatan (Belize, Mexico). I targeted the tips of the most distal branches or plane of the coral, which represent an approximate integration of the C and N sources assimilated during the most recent growth. In Gorgonia, I sampled a continuous patch, approximately 2 x 1 cm from the colony edge. For Pseudopterogorgia, I sampled approximately two branches, 3 – 5 cm in length from the distal end of each specimen. Samples were removed using scissors.
Samples were homogenized into a fine powder by grinding in a Spex Certiprep 6750 Freezer/Mill, followed by a 6-hour glass-thimble soxhlet extraction in 87:13 (volume) chloroform:methanol to remove all lipids. Samples were dried at 60 C for 24 hours. To remove carbonates, the samples were placed in a glass desiccator containing a 12N HCl atmosphere for 48 hours, or until no bubbling was evident from adding concentrated HCl drop-wise to a duplicate sample observed under a dissecting microscope (Hedges & Stern, 1984). Samples were then removed from this treatment, and oven-dried at 60 C for 24 hours. Approximately ~1.5 mg of tissue (coenenchyme, polyps, and axis) was weighed in 4 x 6 mm tin capsules, combusted, and analyzed by a Carlo-Erba NC2500 Elemental Analyzer coupled to a Finnegan – MAT Delta Plus Isotope Ratio Mass Spectrometer by a Conflo II open-split interface operated by the Cornell Stable Isotope Lab (COIL). Reported $\delta^{15}$N and $\delta^{13}$C values are relative to atmospheric N$_2$ and Vienna Pee Dee Belemnite, respectively. Precision was determined by analysis of an in-house isotope standard (‘BCBG’), which is calibrated against IAEA standards, as well as duplicate samples of a homogenized gorgonian standard. Precision of the $\delta^{15}$N and $\delta^{13}$C measurement of standards was better than 0.18‰ and 0.14‰ (s.d.), respectively.

**Results**

*Genus-specific differences in isotope values*

Overall, there was significant variation in both $\delta^{15}$N and $\delta^{13}$C in the gorgonian collection. $\delta^{15}$N values ranged from 1.3 to 7.0‰ with mean of 3.8‰ ($n = 95$). $\delta^{13}$C ranged from -19.4 to -7.8‰ with a mean of -13.9‰. Both $\delta^{15}$N and $\delta^{13}$C varied significantly between genera. *Pseudopterogorgia* ($n = 68$, mean ± SE = 3.9 ± 0.1) was
slightly enriched in $^{15}$N by approximately 0.5‰ relative to *Gorgonia* ($n = 27; 3.4 \pm 0.2$; Student’s t-test, $df = 1, t = 2.03, p = 0.04$). *Pseudopterogorgia* (-14.8 ± 0.2) were, on average, depleted in $^{13}$C relative to *Gorgonia* (-11.7 ± 0.4) by approximately 3.1‰ (Student’s t-test, $df = 1, t = -6.99, p < 0.0001$).

**Genus-specific temporal trends in $\delta^{15}$N and $\delta^{13}$C**

Temporal trends in $\delta^{15}$N and $\delta^{13}$C were not similar between genera (Fig. 4.2). There were significant negative correlations between $\delta^{15}$N and time in *Pseudopterogorgia* (Fig. 4.2a) and *Gorgonia* (Fig. 4.2c). The rate of change in $\delta^{15}$N was equal in both genera (-0.013 ‰ yr$^{-1}$), though there was more variability in *Pseudopterogorgia* than *Gorgonia* ($R^2 = 0.15$ vs. 0.31, respectively). Furthermore, there was a significant negative correlation between $\delta^{13}$C and year of collection in *Pseudopterogorgia* (Fig. 4.2b), but not in *Gorgonia* (Fig. 4.2d). Using data from Francey *et al.* (1999) I tested the hypothesis that the observed decline in $\delta^{13}$C of sea plumes was due to the increase in $^{13}$C depleted anthropogenic CO$_2$. I found a significant negative correlation between $\delta^{13}$C of *Pseudopterogorgia* and atmospheric CO$_2$ concentrations, which explained 30% of the variance (Fig. 4.3). To account for bias due to genus-specific differences, I normalized the data by applying a correction factor to the *Gorgonia* isotope values. All subsequent statistical tests were not affected by this normalization, so the data were left unaltered.
Figure 4.2. $\delta^{15}$N (right panels) and $\delta^{13}$C (left panels) as a function of year of collection for *Pseudopterogorgia* (A, B) and *Gorgonia* (C, D). Mean values were calculated where more than one sample per year was analyzed. Black lines signify significant linear correlations at $\alpha = 0.05$. Note that significant trends were not found in the genera at higher relative trophic levels.
Figure 4.3. Mean δ¹³C of sea plumes (*Pseudopterogorgia*) as a function of atmospheric CO₂ concentrations from 1862-1978 (data from Francey *et al.* 1999).

The linear model fit to our data suggests that δ¹⁵N has declined by ~1.7 ‰ from 1862 to 1995 in *Pseudopterogorgia*, and by ~1.9 ‰ in *Gorgonia* from 1862 - 2005. Given the increase in the quantity and rate of fertilizer use in the Caribbean after 1960, I split the data here for comparison and pooled data from these two genera. There were significant differences between pre- and post-1960 isotope values for both C and N, with relatively enriched values from the pre-1960 samples (Fig. 4.4). Post-1960, mean δ¹⁵N was significantly lower by more than 1.0 ‰ than the pre-1960 mean (Fig. 4.4a). Likewise, mean δ¹³C before 1960 was 1.1 ‰ higher than the mean value of post-1960 samples (Fig. 4.4b).
Figure 4.4 Mean $\delta^{15}\text{N}$ (A) and $\delta^{13}\text{C}$ (B) of *Gorgonia* and *Pseudopterogorgia* samples binned by year of collection before and after 1960. Error bars represent standard error.
A

$\delta^{15}N$ (‰)

4.5

4.0

3.5

3.0

Pre-1960

Post-1960

$\delta^{13}C$ (‰)

-12

-13

-14

-15

$t = -3.92$

$p = 0.0002$

$df = 93$

$t = -2.08$

$p = 0.04$

$df = 93$
Figure 4.5. $\delta^{15}\text{N}$ as a function of year of collection for *Gorgonia* and *Pseudopterogorgia* from 4 major regions represented in the collection. Data shown are pooled from both genera and averaged if more than one sample of either genus was collected in a given year. Black lines signify the linear best fit, though significant relationships were found for data from the Greater Antilles and Yucatan, only.
Geographic variation in $\delta^{15}N$

Using both *Gorgonia* and *Pseudopterogorgia* data, I found that in all 4 regions of the Caribbean (Greater Antilles, Lesser Antilles, North Atlantic, and Yucatan) there were negative trends in $\delta^{15}N$ over time (Fig. 4.5). These trends were statistically significant in 2 out of 4 regions; the Greater Antilles (Fig. 4.5a), and the Yucatan (Fig. 4.5d). On average, the range in $\delta^{15}N$ values among all regions was $\sim 4.3\%$. The rate of change in $\delta^{15}N$ in the Yucatan ($-0.11 \%$ yr$^{-1}$) was nearly double that of the Greater Antilles ($-0.04 \%$ yr$^{-1}$). To test the hypothesis that these significant declines in $\delta^{15}N$ are due to fertilizer use, I ran correlation analysis using fertilizer data from the Food and Agriculture Organization of the United Nations (FAO-STAT). There was a significant negative relationship between annual mean $\delta^{15}N$ and fertilizer consumption in the Yucatan (Fig. 4.6), though fertilizer was not a good predictor of $\delta^{15}N$ in the Greater Antilles ($n = 4, R^2 = 0.46, p = 0.32$).
Figure 4.6. Annual mean $\delta^{15}N$ as a function of fertilizer consumption in the Yucatan region (Mexico and Belize) from 1961 – 2000. Fertilizer data from FAOSTAT.

Discussion

Long-term trends in $\delta^{15}N$ and $\delta^{13}C$

Land-clearing and biomass burning, followed by accelerating fossil fuel use has released sufficient $^{12}CO_2$ to alter the $\delta^{13}C$ of the atmosphere (Druffel & Benavides, 1986, Francey et al., 1999, Freyer & Belacy, 1983, Keeling et al., 1979). Photosynthetic organisms fix this C source into their biomass, thus preserving a record of its presence. Negative trends in $\delta^{13}C$ have been observed in a variety $\delta^{13}C$ of
terrestrial and marine taxa, especially in the last century (Bump et al., 2007, Druffel, 1997). I observed a similar trend in the δ\textsuperscript{13}C of Pseudopterogorgia. I suggest that the decline is indicative of the Suess effect; the accumulation of \textsuperscript{13}C-depleted CO\textsubscript{2} in the biosphere due to the accelerated combustion of terrestrial biomass and fossil fuels. The rate of change in δ\textsuperscript{13}C (Fig. 4.2b; -0.024 ‰ yr\textsuperscript{-1}) was similar to records from monitoring dissolved inorganic C in the surface ocean off Bermuda (Bacastow et al., 1996), and deep-sea antipatherians off the Southeastern USA (Williams et al., 2007). The linear model fit to our data estimates a ~3.1 ‰ reduction in δ\textsuperscript{13}C from the mid-1800s to 1980 (Δ\textsuperscript{13}C). This is slightly greater than the ~2.0 ‰ decline observed in pine trees over the same period (Freyer & Belacy, 1983). The 1.1 ‰ discrepancy in Δ\textsuperscript{13}C between these terrestrial and marine records could be due to increased terrestrial C inputs as a result of Caribbean-wide deforestation and development, which contributes additional \textsuperscript{13}C-depleted sources to the coastal ocean (Druffel & Benavides, 1986). That sea plume δ\textsuperscript{13}C was negatively correlated with increasing atmospheric CO\textsubscript{2} concentrations further supports this hypothesis (Fig. 4.3).

Given the ~20 % increase in anthropogenic C over the last century, and the fidelity of δ\textsuperscript{13}C records to this change, I expected to see a dramatic change in N isotope ratios in light of the global N cycle having been perturbed by more than 90%, primarily from the use of synthetic agricultural fertilizers (Galloway et al., 2004, NRC, 2000, Vitousek et al., 1997). Overall, I detected a significant negative trend in δ\textsuperscript{15}N, which ranged from +1.3 to +7.0 ‰ (Fig. 4.2a, Fig. 4.2c). In the context of isotope ecology this range is close to the average observed difference between two trophic levels within a food web (Minagawa & Wada, 1984). The linear models fit to our data suggests that, on average, δ\textsuperscript{15}N has declined by ~1.8 ‰ from the mid-1800s to 2005. I hypothesize that Caribbean-wide use of \textsuperscript{15}N-depleted synthetic fertilizers is driving this
depletion, though quantitative testing of this hypothesis is challenging given the uncertainty in our dataset due to the likelihood of local variation in N sources. I did not detect an overall relationship between Caribbean fertilizer consumption from 1965 to 1995 and $\delta^{15}$N, which may be a result of mixing of natural and anthropogenic N sources. Nevertheless, I argue that isotopic depletion of the regional N pool by agricultural fertilizers is the most plausible explanation for the observed decline in the $\delta^{15}$N ratios of sea fans and plumes, given the magnitude anthropogenic change of this source relative to other isotopically depleted sources like atmospheric deposition and N fixation, and the direct impacts of land-based runoff on the marine environment.

An increase in other low $\delta^{15}$N sources provides alternative explanations for the observed decline in gorgonian $\delta^{15}$N. N fixation is a process that has relatively no fractionation from source ($N_2$) to sink (organic N). As a result, ecosystems with high rates of N fixation are a source of low $\delta^{15}$N -N, which propagates through the local food web. While $^{15}$N depleted diazotrophs such as cyanobacteria facilitate isotopic depletion in marine food webs, it is not likely fixed N sources have increased over the last 143 years at the same magnitude as fertilizer inputs, nor that sea plumes have gradually shifted their trophic level to exploit isotopically lighter prey. Ren et al., (2009) have recently shown by quantifying $\delta^{15}$N values of sedimentary foraminifera that increasing biological N fixation has lowered the $\delta^{15}$N baseline of bioavailable N in the Caribbean since the last Ice Age. However, within the past ~5,000 years this trend has reversed and $\delta^{15}$N has increased. Furthermore, as N inhibits fixation by reducing the production and activity of the nitrogenase enzyme, increasing coastal N pollution should suppress N fixation (Vintila & El-Shehawy, 2007, Vitousek et al., 2002). Although increasing N fixation contributes reducing the $\delta^{15}$N of the coastal N pool, I
argue that this process was not a likely factor in our observed decline in the $\delta^{15}\text{N}$ of sea fans and sea plumes over the last century.

Another low $\delta^{15}\text{N}$ source comes in the form of particulate and gaseous N from biomass burning, fossil fuel combustion, and volatization of agricultural fertilizers, which are deposited to surface waters. N deposition is a significant pollutant in estuaries and coastal waters of the Northeastern USA. Recent work has shown that the isotopic effects of N deposition are recorded in benthic algae (Barile & Lapointe, 2005). However, whether or not this source has been important historically (i.e. over the last 143 years) is questionable. Galloway et al. (2004) highlight that global N deposition has tripled since the 1860s, though their geographical analyses illustrate that the Caribbean has not been a recipient of this perturbation. In the future atmospheric deposition will become more important as development continues in the Caribbean, and by 2050, atmospheric N deposition could more than double (Galloway et al., 2004). However, given that our samples were collected between 1862 and 2005, the trends I’ve observed in gorgonian $\delta^{15}\text{N}$ are not likely due to N deposition.

Light-mediated isotope effects could also explain a decrease in $\delta^{15}\text{N}$ over time, especially if samples were obtained from deeper waters in recent years. Heikoop et al. (1998) demonstrated that coral $\delta^{15}\text{N}$ is positively correlated with light exposure as evidenced by an average drop of 0.05‰ per meter depth in hard corals from Jamaica and Zanzibar. Over the 29 m depth range sampled in their study, light caused a ~1.4‰ difference between the $\delta^{15}\text{N}$ values of shallow vs. deep corals. In high light environments, photosynthetic rates are elevated which increases the internal demand for N, depletes the internal N pool, and thus eliminates any isotopic fractionation from assimilation to incorporation in tissues (Heikoop et al., 1998). Conversely, in low light
environments photosynthetic rates are reduced, allowing for the build-up of an internal N pool. Selective assimilation of \(^{14}\)N from this pool lowers the \(^{15}\)N of the coral relative to the source. Of those samples with a record of the depth of collection, I could test this hypothesis by using correlation analysis between \(^{15}\)N and depth. I found no significant relationship between \(^{15}\)N and depth for 32 samples of *Gorgonia* and *Pseudopterogorgia* with a depth record (\(R^2 = 0.04, p = 0.22\)). Thus, I refute the hypothesis that the observed negative trend in \(^{15}\)N is a result of sampling deeper corals through time.

Given the relatively low magnitude of increase in N fixation and N deposition Caribbean-wide, and the improbability of sampling bias or increasing trophic positions by these corals, I argue that isotopic depletion of the regional N pool by human perturbation is the most likely explanation for our observed negative trends, and agricultural fertilizers are the cause. That Caribbean-wide fertilizer inputs are sufficient to affect the isotopic ratio of these corals not only suggests a measurable perturbation of N dynamics over the last 50 years but also an increase in the overall size of the N pool in coastal seas since the 1960’s. It cannot be overlooked that during this same period, coral reefs, which are sensitive to nutrient pollution have declined in the Caribbean by 80% (Gardner *et al.*, 2003).

The overall trend in \(^{15}\)N of sea fans and plumes is relatively conserved across major regions of the Western Atlantic with an average \(\Delta^{15}\)N of -4.3‰. Corals from the Greater Antilles and the Meso-American barrier reef of the Yucatan exhibited significant negative trends through time. The Yucatan dataset is especially striking (Fig. 4.5d), with a rapid 4.3‰ decline in \(^{15}\)N in only 38 years, from 1955 to 1993. There was a greater range of values in the Greater Antilles (\(\Delta^{15}\)N = -5.3‰), though the
decline was over 73 years and therefore not as rapid as seen in the Yucatan. It is difficult to test the hypothesis that these declines and the variability seen in the trends is a direct result of isotopic depletion from the addition of agricultural fertilizers due to the lack of historical data on fertilizer use prior to the 1960s and the relative differences in the magnitude of fertilizer used between Caribbean nations. Given the striking change in $\delta^{15}N$ in the Yucatan since the 1950s, and the similar geology, hydrodynamics, and continuity of the reef structure between Mexico and Belize, I obtained fertilizer consumption data from this region and found a strong negative correlation between these data and annual mean $\delta^{15}N$ (Fig. 4.6). Despite the low sample size in this analysis, I cannot ignore the fact that between 1961 and 2000 per capita agricultural land use in Belize and Mexico increased by 156 and 21%, respectively (http://globalis.gvu.unu.edu) while total fertilizer consumed in Belize increased by 1400% (from 0.4 to 6.1 Gg) and in Mexico nearly 900% (from 191 to 1832 Gg; FAO-STAT) over the same period. Therefore it is likely that the cause of isotopic depletion in this region is due to fertilizer use, and that gorgonian corals are recording these changes with high fidelity. This correlation was not observed in the Greater Antilles, which could be due to 1) differences in the temporal and spatial application of fertilizers in the region, 2) variability in population sizes and land use adding noise from isotopically heavy sewage N inputs, or 3) physical differences in the hydrology and geology of the land-sea interface. Of the countries within the Greater Antilles, all have had substantial increases in fertilizer consumption. Between 1961 and 2000, fertilizer consumption increased in Cuba (105 to 130 Gg), Haiti (0.1 to 14.4 Gg), Jamaica (13.5 to 22.4 Gg), and the Dominican Republic (14 to 92 Gg), though it is apparent from these consumption estimates that the overall quantities and magnitude of change varies between these countries. Countries using the least amount of fertilizer during this time may not have had significant depletion in $\delta^{15}N$ of
nearshore marine organisms. Differences in population growth could be adding noise to the $\delta^{15}$N trend from the Greater Antilles, as well as the other regions of this study. Countries with urbanized coastal areas are more likely to have high population densities, and consequently, a higher proportion of sewage derived N in nearshore habitats. Finally, isotopic fractionations associated with denitrification could interfere with temporal isotope records. Regions with river catchments and substantial wetlands are likely to have higher rates of denitrification and removal of N, which would eliminate the depleted signal from fertilizers through fractionation. This may explain why such strong trends are observed in the Yucatan, where surface waters are rare and denitrification in groundwater is limited by high flow rates, low retention times and minimal dissolved organics (ArandaCirerol et al., 2006, Pacheco et al., 2001). As future fertilizer consumption data are made available, these analyses can be refined to further corroborate the impact of fertilizer N on $\delta^{15}$N records.

**Genus specific isotope ratios**

While corals in general are considered “mixotrophs”, having the ability to acquire energy and nutrients from both their symbiotic partners and direct feeding, there appears to be a continuum with respect to the net contributions of heterotrophy that is species specific. For studies such as ours, aimed at quantifying the isotope values of the nutrient sources at the base of a marine food web, analyzing autotrophic organisms is preferential to omnivores because I are looking for change in the nutrient sources to the base of the food web. Animals that can facultatively switch between food sources could show increased noise in isotope values as a result of assimilating nutrients from multiple trophic levels in the food web.
The strongest correlation between $\delta^{15}$N and time was observed in *Gorgonia*, which had a lower mean $\delta^{15}$N than *Pseudopterogorgia*. Moreover, the only significant correlation between $\delta^{13}$C and time was observed in *Pseudopterogorgia*, which had a lower mean $\delta^{13}$C. The slight isotopic differences between these genera are likely an indicator of species-specific nutrition. Sea plumes, having a relatively lower $\delta^{13}$C than sea fans may be slightly more reliant on dietary C, as plankton tend to be depleted in $^{13}$C relative to corals (Land *et al.*, 1975). Furthermore, sea plumes were slightly enriched in $\delta^{15}$N relative to sea fans, which further supports the idea that plumes are nutritionally occupying a slightly elevated trophic level. Future analyses of species-specific patterns of isotope values, as well as isotope tracer feeding experiments will shed light on the relative differences between gorgonian species and help to identify key species to target for environmental records.

*Long term C and N isotope studies*

Several previous studies reconstructing isotopic time-series from few individual specimens across narrow geographic scales have reported a variety of trends and interpretations, likely due to differences in temporal and spatial sampling (Bohm *et al.*, 1996, Druffel & Benavides, 1986, Druffel & Griffin, 1999, Gischler, 2005, Marion *et al.*, 2005, Quinn *et al.*, 1998, Sherwood *et al.*, 2005, Ward-Paige *et al.*, 2005, Williams *et al.*, 2007). All of these published C and N isotope trends from marine invertebrates are based on few specimens collected over a limited spatial scale (Table 4.1). This does not appear to be problematic for studies using $\delta^{13}$C as a direct measure of change within the global and relatively homogeneous atmospheric C pool, as evidenced by the agreement among the directionality of $\delta^{13}$C trends and their interpretation across taxa. However, for published records of coastal nitrogen pollution, localized sources appear
Table 4.1. Summary of long-term studies of marine invertebrate carbon and nitrogen stable isotope trends greater than 20 years, including their direction, temporal span, and interpretation.

<table>
<thead>
<tr>
<th>STUDY (YEAR)</th>
<th>SPECIMEN</th>
<th># ind.</th>
<th>δ(^{13})C Trend</th>
<th>INTERPRETATION</th>
<th>δ(^{15})N Trend</th>
<th>INTERPRETATION</th>
<th>TIMESPAN range cal. yr (max. #yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>This study</strong></td>
<td>Gorgonian</td>
<td>64</td>
<td>↓</td>
<td>Suess</td>
<td>↓</td>
<td>Agricultural fertilizers</td>
<td>1862-2005 (143)</td>
</tr>
<tr>
<td></td>
<td>Gishler &amp; Oschmann (2005)</td>
<td>Scleractinian</td>
<td>4</td>
<td>↓</td>
<td>Suess, cloud cover (offshore), terr. inputs (nearshore)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Nozaki <em>et al.</em> (1978)</td>
<td>Scleractinian</td>
<td>1</td>
<td>↓</td>
<td>Suess, upwelling</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Quinn <em>et al.</em> (1998)</td>
<td>Scleractinian</td>
<td>1</td>
<td>↓</td>
<td>Suess, cloud cover, climate shifts</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Böhm <em>et al.</em> (1996)</td>
<td>Sponge</td>
<td>3</td>
<td>↓</td>
<td>Suess, most severe since 1970</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
to drive isotopic trends within narrowly defined geographic areas. Thus, without sampling at large geographic scales it is possible that general patterns of change are missed or overly generalized. Francey (1981) emphasized this important point and noted that broad geographic assessments are important for evaluating the global impacts of anthropogenic change. Indeed, varied and localized N sources are a possible explanation for the differences among published δ¹⁵N trends and their interpretations. Of the four δ¹⁵N studies involving > 20-year time-series from marine invertebrates, half reported positive δ¹⁵N trends evoking sewage pollution (Williams et al., 2007) and trophic shifts (Sherwood et al., 2005), while the others reported a decline in δ¹⁵N as a result of agricultural fertilizer inputs (Marion et al., 2005). Moreover, these studies rely on reconstructions from few individuals (max n = 4), and to date there have been no studies which have examined multiple, independently sampled individuals across space and time to determine the generality of environmental change from human activities across broad spatial scales. Furthermore, only one of these studies reconstructed a time-series >50 years (Williams et al., 2007), while the others have failed to incorporate specimens prior to the green revolution, an important baseline with respect to anthropogenic N pollution from synthetic fertilizers.

Future isotope monitoring

Our data suggest that a signal of N isotope depletion across the Caribbean basin is detectable in near-shore gorgonians, despite the potential noise contributed by spatial variability from local factors (e.g. atmospheric deposition, N fixation, upwelling, sewage) that influence δ¹⁵N. This is the first evidence of gross perturbations of N in the Caribbean Sea, coincident with the widespread decline of coral reefs. Over the last several decades, Caribbean agriculture has been supplanted by coastal development
for settlement and tourism, raising concern over the consequent issue of wastewater management. By 2030, Caribbean fertilizer use and population are projected to increase by 2.8 and 23 %, respectively (Zhang & Zhang, 2007). Thus, future anthropogenic contributions to the coastal N pool are likely to be dominated by sewage. As sewage-derived N typically has high δ^{15}N values it is probable that in many regions sewage and fertilizer sources will act as opposing end-members of N isotope mixing, which ultimately negates the perceived presence of either source (Lapointe et al., 2004, Risk et al., 2009). It is possible that in rapidly developed regions like the Northern Atlantic and Lesser Antilles where coastal populations and agriculture have grown in concert, that mixing of sewage and fertilizer derived N is the reason for the lack of any trend in δ^{15}N over time (Lapointe et al., 2004).
Figure 4.7. Log-transformed annual mass of N potentially contributed to the Caribbean Sea via fertilizer and sewage as determined by FAO-STAT (left y-axis). Sewage was estimated by assuming a mean nitrogenous waste production of 3.9 kg N person\(^{-1}\) year\(^{-1}\). Grey bars signify the output of a two end-member mixing model:

\[
\delta^{15}N_{\text{Integrator}} = \frac{(kg N_{\text{Fertilizer}} \times \delta^{15}N_{\text{Fertilizer}}) + (kg N_{\text{Sewage}} \times \delta^{15}N_{\text{Sewage}})}{kg N_{\text{Fertilizer}} + kg N_{\text{Sewage}}}
\]

Note that the maximum predicted isotope value occurs during the only interval where sewage contributions are greater than fertilizer.
To predict how shifting land use might affect changes in $\delta^{15}$N, I constructed a simple two end-member mixing model (Fig. 4.7). Excluding variation within natural N sources and assuming a hypothetical integrator of those sources without fractionation, the model uses estimates of the mass of N contributed from fertilizer and sewage and assumed average $\delta^{15}$N of 0 and 8‰, respectively (Heaton, 1986, Mayer et al., 2002) and predicts that a perfect integrator in the Caribbean would have reached an isotopic minimum in the mid-1980’s, with a $\Delta^{15}$N of just 1.6‰, relative to the mid-1960s. The mixing model also illustrates how this trend would reverse as contributions of N from sewage surpass agricultural fertilizer use. With this in mind and based on our observation of historical trends in $\delta^{15}$N, I caution that studies reporting the perceived presence of sewage-derived N by stable isotope methods may be underestimating the magnitude of sewage pollution due to the enormous contribution of $^{15}$N-depleted synthetic fertilizers to coastal waters. Isotopic mixing of anthropogenic sources, as recorded by living organisms, can result in the erroneous interpretation of a “pristine” ecosystem. Therefore, claims of isotopic thresholds for the presence N pollution may be unfounded without a long-term historical context.
REFERENCES


