# A COMPARISON OF PRE-TREATMENT METHODS FOR $\delta^{15}N$ ANALYSIS IN MOLLUSK SHELLS

by

JESTINA ANNE HANSEN

C. FRED T. ANDRUS, CHAIR
RUTH H. CARMICHAEL
RONA J. DONAHOE
ALBERTO PÉREZ-HUERTA

A THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Department of Geological Sciences
in the Graduate School of
The University of Alabama

TUSCALOOSA, ALABAMA

2011

ABSTRACT

Two sample preparation methods, acid pretreatment and no pretreatment, for $\delta^{15}N$ analysis in mollusk shells were compared on sample splits from three common Gulf of Mexico and North Atlantic mollusks (*Mercenaria spp., Crassostrea virginica,* and *Mytilus edulis*). In all but one sample, no statistically significant difference ($2\sigma$) in $\delta^{15}N$ values was measured between these two preparation techniques. However, sample splits that were not acid pretreated produced lighter $\delta^{15}N$ values than their acidified counterparts in 82% of samples studied, and lower N content in small samples correlated with greater differences in method results. In addition, shell biomineralogy directly affected the %N of the samples; calcitic shell material contained greater %N, and produced data with higher analytical precision than aragonitic shell in the analyzed taxa. These data suggest that shell N content controls analytical data precision and that biomineralogy controls shell %N and N content. Within a singe species, N shell content varied as much as 30µg in *C. virginica* and 24µg in *Mercenaria spp.*, likely as a result of differences in available food supply and N sources to grow-out locations. Because %N can vary greatly among and within species, preliminary analyses are recommended to determine the expected N content in samples and to establish whether omitting acid pretreatment of samples will result in sufficient analytical data precision. N content should also be reported along with analytical error to demonstrate that results are robust.

DEDICATION

To my parents, Judith and Martin Hansen, and to my husband and daughter, Scott and Kyrie Anderson; thank you.

## LIST OF ABBREVIATIONS AND SYMBOLS

$\delta^{15}N$ delta notation $\delta^{15}N = \{(R_{sample}-R_{standard}/R_{standard})\}*10^3$ ‰ where $R_{sample}={}^{15}N/{}^{14}N$ ratio of the sample, and $R_{standard}={}^{15}N/{}^{14}N$ ratio of the standard

*IRMS* isotope ratio mass spectrometer

*EA* elemental analyzer

*B2155* protein isotope standard

*B2151* high organic content sediment isotope standard

*1N* one normal, N = normality (gram equivalents/liters solution)

*N* nitrogen

*Ca* calcium

$CO_2$ carbon dioxide

*HCl* hydrochloric acid

$PtCl_2$ platinum chloride

$H_2O$ water

*IRMS* isotope ratio mass spectrometer

*psi* pounds per square inch

‰ per mil

*%* percent

$\sigma$ standard deviation

*mg* milligram

*mm* millimeter

| | |
|---|---|
| $mV$ | millivolt |
| $\mu g$ | microgram |

ACKNOWLEDGEMENTS

I would first like to thank my adviser, Dr. Fred Andrus, for not only immeasurable patience, time, and explanations, but for support and understanding of the dual commitments of newborn family and work. Thanks and love to my husband, Scott Anderson, for unending support and help in sharing those commitments. A great debt goes to my committee member Dr. Ruth Carmichael, who welcomed me into her lab at the Dauphin Island Sea Lab, guided me through the acid pretreatment method, and provided invaluable advice. Thanks also to Miguel Etayo for sharing his work with me, and to Johanna Etayo for taking such wonderful care of our baby girl and for helping me find endless items. Christie Jones' friendship, encouragement, and formatting advice saved my life, especially during MST nights. I appreciate Dr. Jonathon Wynn at the University of South Florida for an amazing rate of sample analysis and technical explanations; I could not have finished without his help. Thanks to Dr. Bob Findlay and Janna Brown for sample analysis and equipment use. I am grateful to Dr. Rona Donahoe and the University of Alabama for scholarship nominations during the first difficult year. Last but not least, I would like to thank Dr. Alberto Perez-Huerta, for insights into biomineralogy and helping me to improve my work.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ILLUSTRATIONS

## 1. Introduction

The study of nitrogen stable isotopes ($\delta^{15}N$) in mollusk shells is increasingly being utilized in environmental science (e.g. O' Donnell *et al.*, 2003; Carmichael *et al*., 2008; Daskin *et al*., 2008; Watanabe *et al*., 2009; Bouillon *et al*., in press). $\delta^{15}N$ in mollusk soft tissues and the organic portion of the shell are affected by anthropogenic influences such as wastewater and fertilizer pollution (e.g. McClelland *et al*., 1997; Carmichael *et al*., 2008; Watanabe *et al*., 2009) and by natural factors such as trophic level (Stapp *et al*., 1999; Post, 2002), and thus may be used as a proxy for these environmental parameters.

Many previous studies have utilized measurements of $\delta^{15}N$ in mollusk soft tissue (e.g. Post, 2002; Weiss *et al*., 2002; Daskin *et al.*, 2008), but this is not a well-preserved environmental record over deeper time. More recent studies developed a method of analyzing N isotopes in the acid insoluble portion of the organic portion of mollusk shells (e.g. O'Donnell *et al*., 2003; Carmichael *et al*., 2008; Daskin *et al*., 2008; Kovacs *et al*., 2010), which is more commonly preserved over long periods of time, often deposited in distinguishable time-dependent increments, and is less susceptible to metabolic turnover (Carmichael *et al*., 2008). Analyzing $\delta^{15}N$ in accreted shell also creates the opportunity for time series based $\delta^{15}N$ research (Hansen *et al*., 2009).

Preparations for shell $\delta^{15}N$ determinations often include decalcification of the shell via acidification prior to analysis. Because shell material typically contains extremely low percent N, reducing the shell powder to its organic components ensures the presence of sufficient N for

precise isotopic measurement and reduces the complications of the overwhelming amount of $CO_2$ that would otherwise be released during sample combustion. The pre-treatment process is comparatively complex, may use upwards of 300-500mg of sample powder, and may require up to one week to complete (Carmichael *et al*., 2008; Carmichael and Kovacs, 2010). Therefore, recent research omitted the acidification step and introduced the bulk sample powder into the elemental analyzer (EA) and isotope ratio mass spectrometer (IRMS) for analysis (Dietz, 2008; Hansen *et al*., 2009; Rowell *et al*., 2010; Versteegh *et al*., 2011).

A comparison between these sample preparation methods has not previously been published, but is needed to facilitate correlations of data between studies. For example, it is necessary to determine if both methods are analyzing the same sources of shell N. In the acid-pretreatment method, as noted by Carmichael *et al*. (2008), only the acid-insoluble component of the organic matrix is analyzed, whereas whole shell analysis includes all possible organic and mineral components. As N does not commonly exchange with Ca in aragonite or calcite, it is assumed that the principle measurable source of N in mollusk shells is the organic matrix. However, previous research into time series $\delta^{15}N$ profiles of Peruvian *Trachycardium procerum* clam shells noted a synchronous increase in %N and a distinct decrease in the apparent organic phase (as measured by Raman spectroscopy), which may suggest that N exists in more than one location in the shell. Although considered negligible, N is present in the acid soluble aspartic acid-rich (aspartic acid formula: $C_4H_7NO_4$) glycoproteins in the organic portion of the shell (Weiner and Hood, 1975; Risk *et al*., 2007). Performing both sample preparation techniques on splits of the same shells, one which analyzes only acid insoluble organic components (acid pretreated) and one which analyzes the bulk shell (non-acid pretreated), will help determine if these methods yield comparable results.

Furthermore, due to the extremely low N content in most mollusk shells, the difference in sample size between the acid pretreatment (up to 1000mg shell powder) and the no pretreatment method (up to ~40mg shell) may have a profound effect on the ability to recover sufficient N in small samples, and thus may affect analytical precision and the practicality of each preparation technique. In addition, it is not yet known whether mineralogy may play a role in shell N content.

To address these concerns, this study performs both sample preparation methods on splits of three of the most commonly-studied mollusk species found along the coast of North America; *Mercenaria spp*. (quahog or hard clam), *Mytilus edulis* (blue mussel), and *Crassostrea virginica* (Eastern oyster). The main goals are to determine: 1) Whether the preparation technique affects the determined $\delta^{15}N$ values; 2) the impacts of small sample size and limited N content, and 3) the effects of shell biomineralogy on shell organic matter and %N content.

## 2. Experimental

### 2a. Species

*Mercenaria spp*. is an infaunal, filter-feeding clam which prefers soft substrates such as mud or soft sand. Its range extends along the eastern coast of the United States to the Gulf of Mexico (Kraeuter and Castagna, 2001). Two species (*M. mercenaria* and *M. campechiensis*) and a natural hybrid exist. All three have aragonitic shells and are visually indistinguishable. *Mercenaria spp.* have previously been used for several isotopic and geochemical studies (e.g. Jones and Quitmyer 1996; Elliot *et al*. 2003; Surge and Walker 2006; Gillikin *et al*., 2005; 2007; Andrus and Crowe, 2008), including some of the first efforts to analyze shell $\delta^{15}N$ (e.g. O'Donnell *et al.*, 2003; Carmichael *et al*., 2004; Carmichael *et al.*, 2008).

*M. edulis* is an epifaunal filter feeding mussel found along the north Atlantic coast of North America and Europe. Its shell is coated by a comparatively thick periostracum and contains two distinctly different layers; the outer layer is composed of calcite which is often blue or purplish in color, and the inner layer is composed of pearly white aragonite (Vander Putten *et al*., 2000). The boundary (miostracum) between these layers is typically crisp. Much chemical and sclerochronological work has previously been conducted on this species. (e.g. Versteegh *et al.*, 2011; Dalbeck *et al*., 2006; Gillikin *et al.*, 2006; Wanamaker *et al*., 2006; 2007).

*C. virginica* is an epifaunal filter feeding oyster found along the Atlantic seaboard. It requires harder substrates such as firm mud, sand, rock, or other shells. Its shell is largely composed of calcite except in areas near muscle attachments where aragonite occurs

(Stenzel, 1963). Previous isotopic research has also been completed on the shell of this species (e.g. Kirby *et al.*, 1998; Andrus and Crowe, 2000; Surge *et al*., 2001; Thompson and Andrus, 2011).

*C. virginica, Mercenaria spp*., and *M. edulis* are all common in archaeological sites, many North Atlantic estuaries, and in sub-fossil deposits and museum collections, making them useful as potential historical archives (O'Donnell *et al*., 2003; Andrus, in press).

**2b. Collection sites**

*Mercenaria spp*. were collected from two different locations (Figure 1). Several specimens were obtained from marshes adjacent to Sapelo Island, GA, a largely undeveloped barrier island. Samples of *Mercenaria spp*., previously analyzed by Carmichael *et al.* (2008), were collected from four estuaries in Cape Cod, MA with varying percent wastewater contribution to N loads (Sage Lot Pond, 4%; Green Pond, 54%; Snug Harbor, 59%; and Childs River, 63%). *M. edulis* samples were raised in open water aquaculture in Orwell Cove off of Prince Edward Island, Canada (Figure 1) and were purchased live from a seafood dealer. *C. virginica* samples, previously analyzed for soft tissue $\delta^{15}N$ by Daskin *et al.*, (2008), were collected from four locations at varying distances (0.07km, 0.50km, 2.18km, and 5.68km) from the Clifton C. Williams wastewater treatment plant in Mobile Bay, AL (Figure 1).


Figure 1. Sample locations for *C. virginica, Mercenaria spp.*, and *M. edulis.*

**2c. Sample preparation**

Whole shells were cleaned of soft tissues and any periostracum (*M. edulis*) by abrading the shell surface with a stiff wire brush and variable speed drill. *Mercenaria spp.* shells from Cape Cod, MA and *C. virginica* shells were ground with an agate mortar and pestle to a fine powder. *Mercenaria spp.* samples from Sapelo Island, GA were milled to 1-2mm depth over the entire ontogeny using a variable speed drill. Two splits were created from each shell sample, and one split was treated with acid prior to isotopic analysis. *M. edulis* shells were bisected and examined in cross-section to determine the depth of the miostracum; as much of each layer as possible was then separately milled with a variable speed drill. Two splits of each layer were created, and one of each pair of splits was treated with acid prior to analysis.

i. *Acid Pretreatment*

A solution of 1% $PtCl_2$ in 1N HCl was added gradually to powdered *C. virginica* and *Mercenaria spp.* samples (300mg and 400-550mg, respectively). After each addition, the reaction was allowed to completely finish before more acid was added. Acid digestion was deemed complete when little-to-no carbonate particles remained and no bubbling was observed. The time needed for total acid digestion varied from 3 days for drilled samples to 6 days for hand-ground samples.

*M. edulis* samples ranged in size from 250-400mg for the calcitic layer and 300-400mg for the aragonitic layer. Samples were treated with 1N HCl added in 1ml increments, with more acid added when effervescence became minimal. All samples were gently agitated between acid additions. The finer particle size increased the speed of the reaction, and the time required for total acid digestion was decreased to 1-2 days.

Once acid digestion was complete, the remaining spaces in the sample vials were filled with nanopure $H_2O$ and decanted onto pre-ashed glass microfiber filters, with vacuum suction set at approximately 5 psi to avoid pulling small organic particles through the filter. Filters were rinsed with a minimal amount of ultra pure water. Samples were then dried for at least 8 hours at 50-60$^{\circ}$C. The back layer of the filter was peeled and removed, and the remaining filters with the organic materials were folded and packed in tin capsules for elemental and isotope ratio analysis.

ii. *No Pretreatment*

For each species, 34-35mg of ground sample was weighed and packed into tin capsules for analysis. Each powdered sample was weighed from the same individual as the corresponding acid-pretreated sample.

iii. *Isotopic Analysis*

All samples were analyzed for N isotopes using a Costech ECS Elemental Analyzer with "zero-blank" autosampler coupled to a Thermo (Finnigan) Delta V 3 keV isotope ratio mass spectrometer (IRMS) at the University of South Florida. For non-acidified samples, a carbon trap was fitted between the EA column and IRMS to capture the $CO_2$ produced by the combustion of shell carbonate, allowing the smaller N peaks to be clearly detected. Three standards were used: Acetinalide for %N, a sediment standard (B2151) for $\delta^{15}N$ controls on non-acidified shell and tissue samples, and a protein standard (B2155) for $\delta^{15}N$ control on acidified samples. A range of samples sizes was used for isotope standard B2155 (~0.4 to 0.9mg, correlating to ~40 to 140µg N, respectively) to capture the variation in analytical precision based on total N content. For small sample sizes with low N content (defined by standards with N contents of 48-67 µg), one sigma error was ±0.71‰. For larger samples with high N content (includes acidified samples, calcite samples, and standards with N content 115-140µg), one sigma error was ±0.09‰.

## 3. Results

### 3a. Effect of Pretreatment on $\delta^{15}N$

No significant difference in $\delta^{15}N$ was measured between the acid pretreated and the non acid pretreated samples of *C. virginica*, *Mercenaria spp*., or *M. edulis*. $\delta^{15}N$ values of sample splits were within 2σ error of each other with only one exception (Figures 2- 4). At 1σ, however, there were significant differences between results of the two pretreatment techniques in 10 of 21 *Mercenaria spp*. and 3 of 6 aragonite layer samples of *M. edulis* (Table 1).

Across all species, $\delta^{15}N$ values measured using the non acid pretreatment method were lighter (within 2σ) than for their acidified counterparts in 82% of samples.

### 3b. Effect of N Content on $\delta^{15}N$

Low sample N content resulted in a greater difference between $\delta^{15}N$ results of each pretreatment technique (Figure 5); 100% of samples which recorded a significant difference between pretreatment techniques (at 1σ) contained less than 50μg N. Measurements of sample N content were made only in non-acid pretreated samples, as final sample weights (after acidification) were not known for acid pretreated samples. Although %N affected the analytical accuracy and precision of shell $\delta^{15}N$ measurements, there was no distinct linear correlation between %N and $\delta^{15}N$ ($r^2$=0.022) (Figure 6).

### 3c. Effect of Biomineralogy on %N and Pretreatment Comparisons

Calcite shells contained a greater %N than aragonite across all species (Table 2). Calcite samples therefore yielded higher recovered N content and correspondingly lower differences in $\delta^{15}N$ results between the two pretreatment methods; all paired calcite $\delta^{15}N$ results are within $1\sigma$ error (see Table 1).

### 3d. Anthropogenic Influences on $\delta^{15}N$

Increasing distance from the Clifton Williams WTP correlated with increasingly heavy $\delta^{15}N$ in the *C. virginica* collected near the treatment plant in Mobile Bay, AL (Table 3). Increasing percent contribution to total estuarine N load correlated with heavier $\delta^{15}N$ in the *Mercenaria spp.* collected from four estuaries in Cape Cod, MA (Table 4).

### 3e. Sample Combustion and Analytical Precision

During analysis, observed samples in the elemental analyzer showed flash combustion. N peaks were sharp, and blanks that were run directly after unacidified samples had N levels below the detection limit of 50mV (Appendix A), indicating complete combustion. It should be reiterated that two estimates of analytical precision were made as described in *Methods* to account for variations in overall N content of the samples, and that non acid pretreated samples contain the least N and are therefore associated with the greatest error.

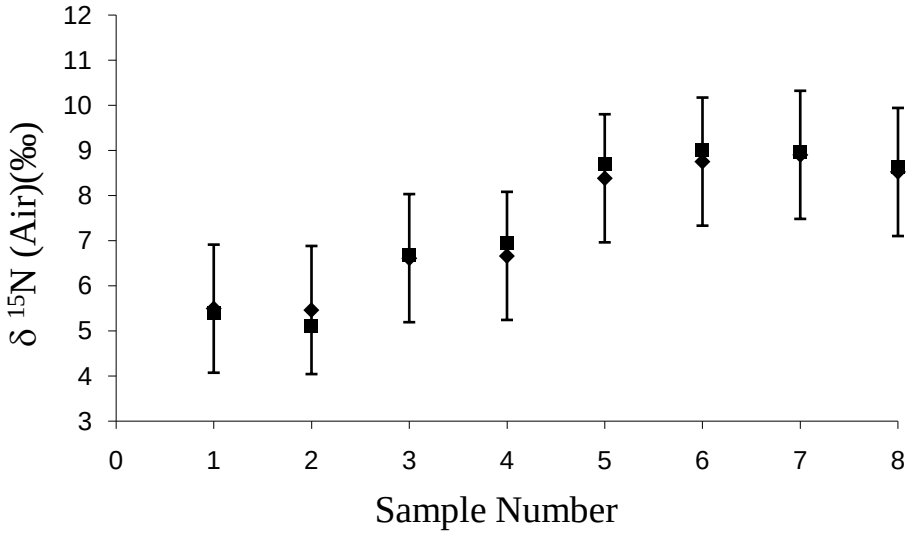


Figure 2. Measured $\delta^{15}N$ values for both acid pretreatment (squares) and non acid pretreatment (diamonds) methods for *C. virginica.* 2σ error bars are shown on unacidified samples. Acid pretreated sample error bars are omitted as they are the same size as the symbols (2σ = 0.18‰) Note the overlap of $\delta^{15}N$ values measured using each technique at the 2σ level.

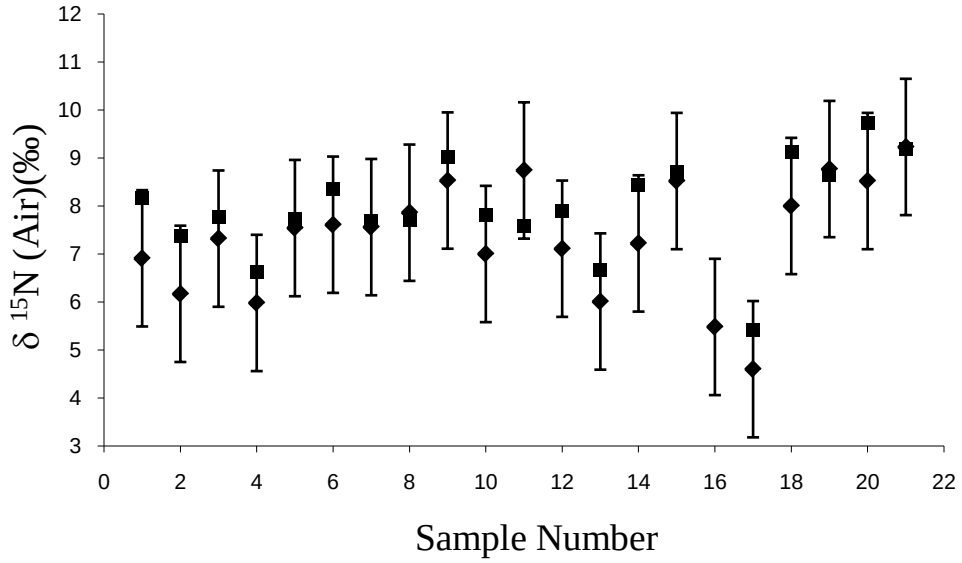


Figure 3. Measured $\delta^{15}N$ values for both acid pretreatment (squares) and non acid pretreatment (diamonds) methods for *Mercenaria spp.* 2σ error bars are shown for non acid pretreatment samples. Acid pretreated sample error bars are omitted as they are nearly the same size as the symbols (2σ = 0.18‰). Note the overlap of $\delta^{15}N$ values measured using each technique at the 2σ level.

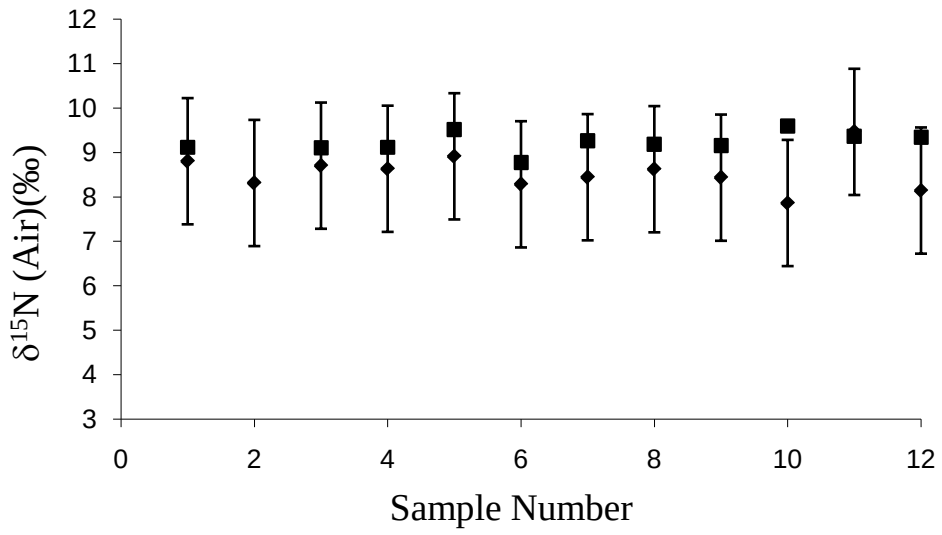


Figure 4. Measured $\delta^{15}N$ values for both acid pretreatment (squares) and non acid pretreatment (diamonds) methods for *M. edulis* calcite (samples 1-6) and aragonite (samples 7-12) layers. 2σ error bars are shown for non acid pretreatment samples. Acid pretreated sample error bars are omitted as they are the same size as the symbols (2σ = 0.18‰). Note the overlap of $\delta^{15}N$ values measured using each technique at the 2σ level.

Table 1. %N and N content for non acid pretreated samples and $\delta^{15}N$ values for both methods. The right-hand column denotes whether $\delta^{15}N$ values for both methods are within 1σ error. N content is approximate and was calculated using a mass of 35mg but actual mass varies as 35±1mg.

| Sample I.D. | $\delta^{15}N$ (Air)(‰) No Pretreatment | $\delta^{15}N$ (Air)(‰) Acid pretreatment | %N | N Content (µg) | Within 1σ? |
|---|---|---|---|---|---|
| *Mercenaria* 1 | 6.91 | 8.16 | 0.04 | 14 | No |
| *Mercenaria* 2 | 6.17 | 7.38 | 0.04 | 14 | No |
| *Mercenaria* 3 | 7.32 | 7.77 | 0.05 | 18 | Yes |
| *Mercenaria* 4 | 5.98 | 6.63 | 0.11 | 39 | Yes |
| *Mercenaria* 5 | 7.54 | 7.72 | 0.06 | 21 | Yes |
| *Mercenaria* 6 | 7.61 | 8.36 | 0.07 | 25 | No |
| *Mercenaria* 7 | 7.56 | 7.68 | 0.10 | 35 | Yes |
| *Mercenaria* 8 | 7.86 | 7.70 | 0.06 | 21 | Yes |
| *Mercenaria* 9 | 8.53 | 9.03 | 0.07 | 25 | Yes |
| *Mercenaria* 10 | 7.00 | 7.81 | 0.05 | 18 | No |
| *Mercenaria* 11 | 8.74 | 7.58 | 0.07 | 25 | No |
| *Mercenaria* 12 | 7.11 | 7.91 | 0.07 | 25 | No |
| *Mercenaria* 13 | 6.01 | 6.66 | 0.08 | 28 | Yes |
| *Mercenaria* 14 | 7.22 | 8.44 | 0.04 | 14 | No |
| *Mercenaria* 15 | 8.52 | 8.70 | 0.07 | 25 | Yes |
| *Mercenaria* 16 | 5.48 | Lost | 0.07 | 25 | Yes |
| *Mercenaria* 17 | 4.60 | 5.42 | 0.06 | 21 | No |
| *Mercenaria* 18 | 8.00 | 9.13 | 0.08 | 28 | No |
| *Mercenaria* 19 | 8.77 | 8.64 | 0.05 | 18 | Yes |
| *Mercenaria* 20 | 8.52 | 9.72 | 0.06 | 21 | No |
| *Mercenaria* 21 | 9.23 | 9.18 | 0.07 | 25 | Yes |
| *C. virginica* 1 | 5.49 | 5.41 | 0.24 | 84 | Yes |
| *C. virginica* 2 | 5.46 | 5.10 | 0.29 | 102 | Yes |
| *C. virginica* 3 | 6.61 | 6.68 | 0.27 | 95 | Yes |
| *C. virginica* 4 | 6.66 | 6.95 | 0.26 | 91 | Yes |
| *C. virginica* 5 | 8.38 | 8.70 | 0.33 | 116 | Yes |
| *C. virginica* 6 | 8.75 | 9.01 | 0.31 | 109 | Yes |
| *C. virginica* 7 | 8.90 | 8.97 | 0.27 | 95 | Yes |
| *C. virginica* 8 | 8.52 | 8.63 | 0.25 | 88 | Yes |
| *M. edulis* 1C | 8.80 | 9.12 | 0.20 | 70 | Yes |
| *M. edulis* 2C | 8.31 | Lost | 0.20 | 70 | Yes |
| *M. edulis* 3C | 8.70 | 9.10 | 0.15 | 53 | Yes |
| *M. edulis* 4C | 8.63 | 9.12 | 0.21 | 74 | Yes |
| *M. edulis* 5C | 8.91 | 9.51 | 0.15 | 53 | Yes |
| *M. edulis* 6C | 8.28 | 8.77 | 0.18 | 63 | Yes |
| *M. edulis* 1A | 8.44 | 9.26 | 0.10 | 35 | No |
| *M. edulis* 2A | 8.62 | 9.18 | 0.09 | 32 | Yes |
| *M. edulis* 3A | 8.43 | 9.15 | 0.09 | 32 | No |
| *M. edulis* 4A | 7.86 | 9.59 | 0.08 | 28 | Yes |
| *M. edulis* 5A | 9.46 | 9.36 | 0.10 | 35 | Yes |
| *M. edulis* 6A | 8.14 | 9.34 | 0.10 | 35 | No |

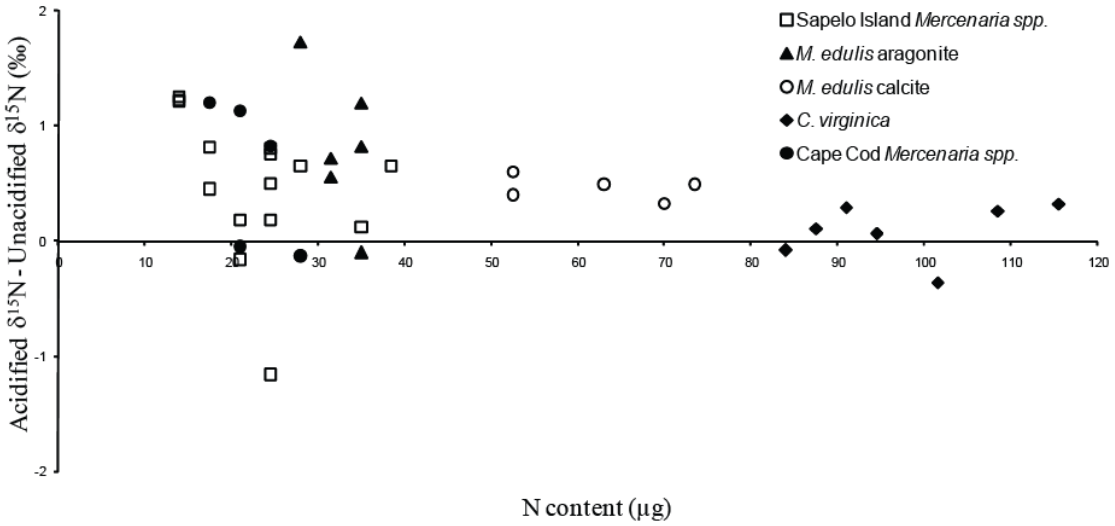


Figure 5. Differences in measured $\delta^{15}N$ between acid pretreatment and non-acid pretreatment methods plotted against recovered N content of each sample for all species.

Table 2. Mean %N, mean N content, and mean difference between $\delta^{15}N$ results for each method, for *C. virginica*, *Mercenaria spp.*, and each layer of *M. edulis.* Only sample pairs in which unacidified samples are lighter than non-acid pretreated samples are accounted for in $\delta^{15}N$ statistical data.

| | ***C. virginica*** | ***Mercenaria spp.*** | ***M. edulis* aragonite** | ***M. edulis* calcite** |
|---|---|---|---|---|
| Mean %N (unacidified samples) | 0.28 | 0.07 | 0.09 | 0.18 |
| Mean N content (unacidified samples) | 95µg | 22µg | 33µg | 63µg |
| Range of differences between $\delta^{15}N$ results for each method | 0.07-0.32‰ | 0.12-1.25‰ | 0.56-1.73‰ | 0.32-0.60‰ |
| Mean difference between $\delta^{15}N$ results for each method | 0.19‰ | 0.74‰ | 1.01‰ | 0.46‰ |

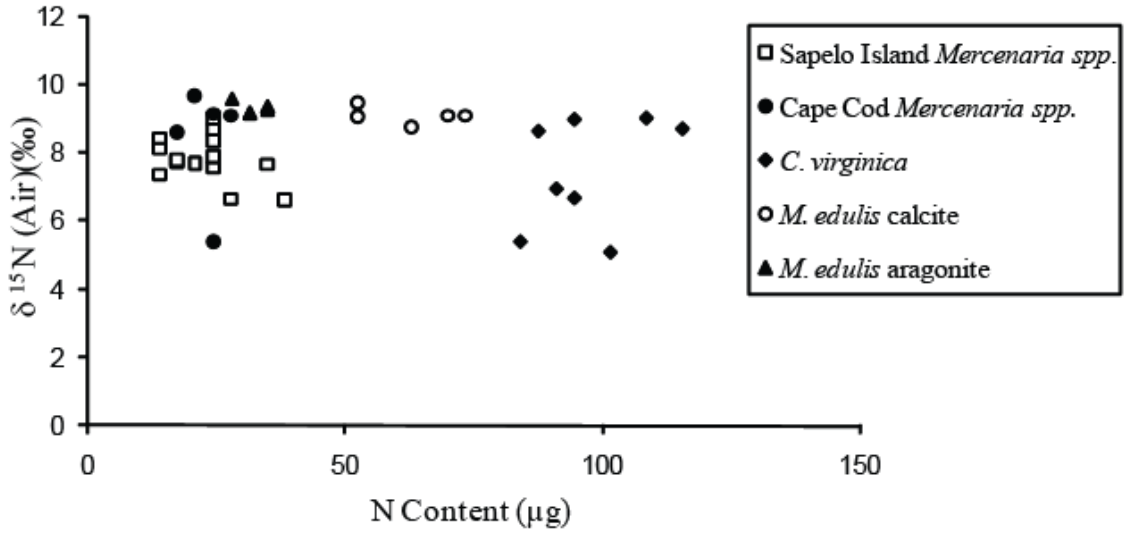


Figure 6- $\delta^{15}N$ plotted against %N for all samples across all species. No pronounced linear correlation between $\delta^{15}N$ and %N is observed for combined species ($r^2$=0.022). R-squared values for each species alone are as follows: *Mercenaria spp.* $r^2=2e^{-5}$; *C. virginica* $r^2$=0.150; *M. edulis* $r^2$=0.042.

Table 3. Average $\delta^{15}N$ of *C. virginica* samples collected from Mobile Bay, AL as compared to distance from the Clifton C. Williams wastewater treatment plant.

| **Sample Name (Sample I.D. #)** | **Distance from Clifton Williams WTP** | **Average $\delta^{15}N$ of Acid Pretreated Samples (‰)** |
|---|---|---|
| Site 1 (1-2) | 0.07km | 5.25 |
| Site 2 (3-4) | 0.50km | 6.82 |
| Site 3 (5-6) | 2.18km | 8.85 |
| Site 4 (7-8) | 5.68km | 8.80 |

Table 4: Average $\delta^{15}N$ of *Mercenaria spp.* samples collected from Cape Cod, MA as compared to percent wastewater contribution to total estuarine N load. Sample I.D. numbers correlate to Table 1.

| **Sample Location (Sample I.D. #)** | **Wastewater Contribution to Total N Load** | **Average $\delta^{15}N$ of Acid Pretreated Samples (‰)** |
|---|---|---|
| Childs River (20) | 63 % | 9.72 |
| Snug Harbor (21) | 59 % | 9.18 |
| Green Pond (18-19) | 54 % | 8.89 |
| Sage Lot Pond (16-17) | 4 % | 5.42 |

## 4. Discussion

There are two trends discovered here to consider: 1) the $\delta^{15}N$ of the unacidified split (low N content) is lighter than its acidified counterpart in 82% of samples (Table 1), and 2) lower N content results in a greater difference in measured $\delta^{15}N$ between methods (Figure 5). It has been previously established that low N content can contribute to analytical errors of anomalously light $\delta^{15}N$ values (e.g. Carmichael *et al.*, 2008 working on samples similar to those described here). Because analytical error in this study is larger for those samples with low N content, and those samples with large error are lighter than those with less error (high N content samples, i.e. acid pretreated), analytical error associated with low N samples is likely the source of both of these trends. In addition, since N content was lower in the aragonite samples than in calcite samples, the different responses to the methods comparison (at the $1\sigma$ level) of the calcite versus aragonite species may be attributed to variations in %N, and consequently analytical error, between species.

### 4a. Analytical Precision

Analytical precision is greatest when N content of samples is well within the accepted detection range of the IRMS (minimum 50mV). In the no-pretreatment preparation method, low sample volume becomes an issue because the sample is introduced as the complete powder with low %N. Limits to sample size may vary between laboratories due to combustion efficiency (e.g. Dietz, 2008 uses 40-90mg ground bivalve shell sample, although does not address the combustion question);

however in most cases, low N content must be a serious concern when using the no-pretreatment preparation method. In addition, the sensitivity of the IRMS used will have an effect on precision, and recent publications have obtained reasonable analytical precision using the non acid pretreatment method with samples containing ~20μg N (Versteegh *et al*., 2011). However, previous studies on *Mercenaria spp.* found that a N content of at least 70-80μg is necessary for accurate $\delta^{15}N$ results (Carmichael *et al.*, 2008). The data presented here more closely agree with this N content requirement as sample pairs containing at least 50μg of N have measured $\delta^{15}N$ values within one sigma error.

Analytical precision is generally greater for the acid-pretreatment method because the dissolution of carbonate removes the majority of the sample's volume and leaves only a greatly decreased amount of more N-rich organics for analysis. Studies using up to 1000mg of pre-dissolution sample have been completed successfully (Kovacs *et al*., 2010).

**4b. Shell N**

Due to the lack of exchange between Ca and N, it is currently assumed that the only significant source of N resides in the organic content of the shell. However, key details regarding biomineralization processes and physical distributions of organic matter are not completely understood (e.g. Cusack and Freer, 2008; Pouget *et al*., 2009). For instance, if a measurable source of N is located in shell components other than the acid insoluble fraction, it is possible that different pathways and fractionation patterns between different organic phases would result in disparate $\delta^{15}N$ signatures between acid pretreatment and non acid pretreatment methods of shell analysis. Recent research has raised the question of whether N may reside in shell components other than the acid insoluble organic portion (Hansen, 2009).

For example, previous time series $\delta^{15}N$ on two analogous Peruvian *Trachycardium* procerum shells found an increase in %N at the same time as a decrease in apparent organic content as determined by Raman spectroscopy (Hansen *et al*., 2009; Etayo *et al*., 2010) (Appendix A). These data may at first suggest that the distribution of N and organic matter in the shell is more complex than previously thought. However this study, which compares $\delta^{15}N$ values of the organic portion with the bulk shell, does not find any difference in measured $\delta^{15}N$ signatures between methods. These results indicate that either both methods capture all sources of N in the shell or that all N sources in the shell record a similar $\delta^{15}N$ signature.

It should be noted that the mollusks in this study did not seem to undergo any stresses on the magnitude of El Niño which the *T. procerum* shells survived, and it is not yet known whether major stresses can affect the incorporation of N into organic shell material. It is worth noting that the %N curve follows the shape of organic content curve prior to El Niño, but deviates afterward, suggesting that the stress of El Niño could be the reason for the anomalous pattern seen after El Niño in the *T. procerum* shells.

In contrast to a natural explanation for the anomalies in the *T. procerum* %N curve, the results of this methods comparison highlight another possibility; since it was a time series analysis and sample was therefore limited, the *T. procerum* data was obtained using the non-acid pretreatment method and small sample volumes of approximately 40-45mg. If one examines the %N curve and calculates the N content of the samples, one will notice that even when %N is reported at its highest, the N content of the samples falls well below the ~50μg minimum. The contrast between the expected relationship between %N and apparent organic content may therefore be partially a result of analytical error.

The *T. procerum* study is an excellent example of why it will be necessary for future authors to report the N content of their samples in order to allow the reader to establish accuracy of data.

**4c. Variations in %N**

Predicting the N content of a given sample requires comparing shell %N and sample weight. However, the amount of organic material in bivalve shells can vary to up to 5 wt% of the shell (Marin and Luquet, 2004; Bouillon *et al*., in press), and preliminary analysis may be required for species in which an average %N has not already been established.

Furthermore, the variation in %N within a single species seen here (0.08-0.21% in *M. edulis*, 0.04-0.11% in *Mercenaria spp*., and 0.24-0.33% in *C. virginica*) indicate that it may be prudent to determine %N of each sample pool regardless of whether an average %N for the particular species has been established. Percent N may also vary as a function of anthropogenic N load and eutrophication effects on food quantity and quality, such as was found in the previous work on the *Mercenaria spp.* and *C. virginica* used in this study (Carmichael *et al*., 2008; Daskin *et al*., 2008), further complicating the issue of predetermining expected N content.

For species in which more than one mineralogy is present (as in *M. edulis*), the %N of each should be determined as it may be sufficient in one mineralogy but not in the other. For instance in the species in this study, aragonite is shown to contain as little as half the %N of the calcite portion of the same shell (as in *M. edulis*).

In some cases, %N may be expected to vary within a single individual and single mineralogy. This was the case for the Peruvian *T. procerum* shell discussed above. Previous research has also shown that changes in food quantity and quality throughout a clam's life can

result in changes in %N throughout ontogeny (Carmichael *et al*., 2004). In a situation where %N and expected N content cannot be pre-determined, it is recommended to use the acid pretreatment method. Although recent studies use several hundred milligrams powder per sample (Kovacs *et al*. 2010, this study), it is possible to use less sample if necessary (such as to allow for time series data from a single shell). Functional $\delta^{15}N$ values have been obtained using as little as 80 mg of sample from *Mercenaria spp.* (Carmichael *et al*., 2008).

**4d. Influence of Anthropogenic Pollution**

The influence of anthropogenic pollution on shell $\delta^{15}N$ found here is the same as noted in previous research; the closer the proximity to the wastewater treatment plant and the greater the wastewater contribution to total estuarine N load, the more the shell $\delta^{15}N$ values trend toward the $\delta^{15}N$ signature of the contamination (Tables 4 and 5). In *Mercenaria spp.*, the wastewater contaminating the shell estuaries was conveyed through groundwater and had a heavier than ambient $\delta^{15}N$ signature (Carmichael *et al*., 2008), and shell $\delta^{15}N$ became heavier with greater percent wastewater contribution. In *C. virginica*, the pollution was largely raw discharged sewage and therefore had lighter than ambient $\delta^{15}N$ (Daskin *et al*., 2008), and samples collected close to the treatment plant recorded lighter shell $\delta^{15}N$ than those collected further away.

**4e. Possible Sources of Error**

**i. *Complete Combustion***

In unacidified samples with low %N, large sample volumes will yield the greatest N content and accuracy. However, sample size is limited by the simple mechanics of how much powder will fit in the capsules and by the concern of complete combustion. Incomplete

combustion could result in analysis of different fractions of the shell samples, thus biasing the results. The possibility of incomplete combustion was assessed in these analyses because the large samples in this study (non acid pretreated) display more negative $\delta^{15}N$ values than their smaller acid-pretreated splits. However it does not seem likely that incomplete combustion is the cause of the consistent negative $\delta^{15}N$ offsets in the non-pretreated samples. Measured N peaks were sharp and blanks that were run directly after unacidified samples had N levels below the detection limit of 50mV (Appendix A), indicating that all sample material was combusted before admission into the IRMS.

**ii. *Sample preparation***

In addition to the difference in acid application between methods, there is one other preparation step that varied between samples: the method of powdering the shells. *C. virginica* and *Mercenaria spp.* from Cape Cod, MA, were powdered using a glass blender and mortar and pestle, whereas *M. edulis* and *Mercenaria spp.* from Sapelo Island, GA were powdered using a variable speed hand drill. This second powdering technique produces greater friction and heat, and breaks down the powder into smaller particles, and may therefore create a possibility of greater fractionation associated with the mechanical breakup of the shell and localized combustion at the point of drilling. However, the fact that all the samples with sufficient N to overcome analytical precision errors exhibit no significant change in $\delta^{15}N$ values between methods suggests that there is at least no detectable difference in $\delta^{15}N$ resulting from the variation in powdering procedure.

**4f. Selecting a Method**

Current research into $\delta^{15}N$ of mollusk shells focuses largely on changes in $\delta^{15}N$ over time or as compared between two sample populations. Therefore, although the non-acid pretreatment method results in $\delta^{15}N$ values generally lower than the acid pretreatment method, it still may be valid for relative comparisons when i) The method used for each sample within the study is consistent, ii) the minimum N content requirement is met (50μg in this study) and iii) analytical standards used are of a similar size to best assess precision. The results of this study show a distinct, significant decrease in the precision and accuracy of the non-acid pretreatment technique in samples containing less than ~50μg N, indicating that this method should not be used in studies where expected N content is below this limit.

Determining which method is most appropriate also depends upon the requirements and goals of a given study. Since samples containing sufficient N content have similar $\delta^{15}N$ for both methods, the results here suggest that the application of 1N HCl does not significantly affect measured $\delta^{15}N$, but does eliminate the concern of low N content by allowing larger sample volumes. Therefore for research in which large sample volumes are possible, the acid pretreatment method is recommended to avoid the complications of analytical error.

However for studies where sample availability is limited and small sample volumes are necessary regardless of analysis method (e.g. to obtain time-series data from a small shell), it may be preferable to use the non-pretreatment method to avoid the time, cost, and increased steps (and thus potential for human error) of the acidification technique. In this case, it becomes vital to determine the expected N content of samples and to use an appropriate variety of standard volumes to scale analytical precision to reflect N content, and to therefore measure the robustness of resultant data.

### 4g. Comparing Data Sets Across Studies and Methods

Perhaps of equal importance to delineating the advantages and pitfalls of each technique, this study also illustrates the caution that must be used when comparing results between studies which used different analysis techniques. For example, there has been much research into the effects of anthropogenic pollution on shell $\delta^{15}N$ (e.g. McClelland *et al*., 1997; Carmichael *et al*., 2008; Daskin *et al*., 2008; Watanabe *et al*., 2009). The discovered correlation between pollution and shell $\delta^{15}N$ will undoubtedly lead to many future studies, some of which may analyze shells from the same location as previous research and attempt to corroborate data. In such a case, it will be vital to know the method used to obtain $\delta^{15}N$ of the shell. A difference in analysis technique may eliminate or create offsets in $\delta^{15}N$ which have little to do with natural fractionation processes.

On a similar note, it is essential for authors to report recovered N content of their shells. If sample volume is not known and %N and N content cannot be calculated (as is often the case in the most recent development of the acid-pretreatment method), authors should report the N peak voltage of their samples and relate it to the detection limit of the IRMS used. In either case, some stringent measurement of accuracy and precision should be included in publications.

## 5. Conclusions

Comparison of the non-acid pretreatment and the acid pretreatment methods of analyzing $\delta^{15}N$ in the shells of *C. virginica, Mercenaria spp.*, and *M. edulis* revealed almost no significant difference at the 2σ level between methods results. However, the two techniques recorded significantly different $\delta^{15}N$ values in approximately half of the aragonite samples at the 1σ level. In addition, a distinct trend exists in which 82% of total samples resulted in lighter $\delta^{15}N$ values in non-acid pretreated samples than in their acid pretreated pairs. These trends are interpreted as being due to insufficient N content in small sample sizes (such as required in non acid pretreated samples) and in aragonite samples, causing increased analytical error (previously determined to often be expressed as an artificial lightening of $\delta^{15}N$ values).

Analytical error in the non acid pretreated method is of lesser concern in samples with sufficiently high N content (e.g. in this study, samples with ≥0.50μg N had $\delta^{15}N$ values for both methods within 1σ error). There is a clear correlation in the species analyzed here between shell biomineralogy and %N, where calcite contains greater %N than aragonite. Therefore, although roughly half of the aragonite shells had different $\delta^{15}N$ reported for each method at the 1σ significance level, none of the calcite samples showed any difference even at 1σ. This indicates that biomineralogy may play an important role in %N of shells and thus on the extent of analytical error.

Since low N content greatly decreases analytical precision and accuracy and leads to significant differences in measured shell $\delta^{15}N$ between the acid pretreatment and non-acid

pretreatment methods, it is essential to determine expected sample N content prior to selecting an analysis technique. Low N samples should be analyzed with the acid pretreatment method, which concentrates N-bearing proteins in the shell by dissolving the calcium carbonate. When attempting to corroborate research, it will be crucial to know which analysis technique was used for each project as well as the N content of samples so that there can be an accurate assessment of potential causes for any differences in shell $\delta^{15}N$. Consequently, authors are strongly encouraged to report both details of analysis techniques and recovered sample N content. Results from species which were collected from estuaries with varying levels of influence from wastewater confirm that shell $\delta^{15}N$ is a valid proxy for anthropogenic pollution. Higher percent wastewater contribution to total estuarine N load resulted in $\delta^{15}N$ signatures in shells that were skewed toward the wastewater values. This correlation reinforces the importance of shell $\delta^{15}N$ studies in upcoming pollution monitoring research, and the determination of analysis techniques and associated precautions is a necessary step in developing the potential of shell $\delta^{15}N$ research.

**References**

Andrus, C. F. T. and D. E. Crowe, 2000, Geochemical analysis of *Crassostrea virginica* as a method to determine season of capture: Journal of Archaeological Science, v. 27, p. 33-42.

Andrus, C.F.T. and D. E. Crowe, 2008, Isotope analysis as a means for determining season of capture for Mercenaria, *in* Thomas, D.H., ed., Native American landscapes of St Catherine's Island, Georgia II: American Museum of Natural History Anthropological Papers 88, p. 498-518.

Andrus, C.F.T. *et al*., 2005, Molluscan radiocarbon as a proxy for El Nino-related upwelling variation in Peru, *in* Surge, D., and Mora, G., eds., Isotopic and Elemental Tracers of Late Quaternary Climate Change: Geological Society of America Special Paper 395, p. 13-19.

Andrus, C. F. T., in review, Isotope Sclerochronology in Southeastern US Archaeology to Estimate Season of Capture, *in* Reitz, E.J., Quitmyer, I.R., and Thomas. D.H., eds., Seasonality of Mobility on the Georgia Bight: Methodologies and Substantive Applications: American Museum of Natural History Anthropological Papers

Bouillon, S., Connelly, R.M., and Gillikin. D.P., in review, Use of stable isotopes to understand food webs and ecosystem functioning in estuaries, *in* Wolanski and McLusky, eds., Treatise on Estuarine and Coastal Science 7

Carmichael, R.H., and Kovacs, C.J, 2010, Comment on Watanabe *et al*. (2009): Marine Pollution Bulletin, v. 60, p. 314-315

Carmichael, R.H. *et al*., 2004, Changes in shell and soft tissue growth, tissue composition, and survival of quahogs, *Mercenaria Mercenaria*, and softshell clams, *Mya arenaria*, in response to eutrophic-driven changes in food supply and habitat: Journal of Experimental Marine Biology and Ecology, v. 313, p. 75-104.

Carmichael, R.H. *et al*, 2008, Nitrogen stable isotopes in the shell of *Mercenaria mercenaria* trace wastewater inputs from watersheds to estuarine ecosystems: Aquatic Biology v. 4, p. 99-111.

Cusack, M. and Freer, A., 2008, Biomineralization: elemental and organic influence in carbonate systems: Chemical Reviews, v. 108, no. 11, p. 4433-4454.

Dalbeck, P. *et al*., 2006, Crystallography and chemistry of the calcium carbonate polymorph switch in *M. edulis* shells: European Journal of Mineralogy, v. 18, no. 5, p. 601-609.

Daskin, J.H. *et al*., 2008, Use of N stable isotope and microbial analyses to define wastewater influence in Mobile Bay, AL: Marine Pollution Bulletin, v. 56, p. 860-868.

Dietz, R.D., 2008, Nitrogen isotopes in bivalve shells from the Colorado River estuary: Evaluating a proxy for changes in riverine nutrient delivery [M.Sc. Thesis]: Tucson, University of Arizona, 38 p.

Elliot, M. *et al*., 2003, Environmental controls on the stable isotopic composition of Mercenaria mercenaria: potential application to paleoenvironmental studies: Geochemistry Geophysics Geosystems, v. 4, p. 1–16.

Etayo-Cadavid M.F., 2010, Peruvian mollusk shells as multi-proxy archives: late Holocene upwelling variation and El Niño-induced biomineralization effects on trace elements [Ph.D. dissertation]: Tuscaloosa, University of Alabama.

Gillikin, D. P. *et al*., 2005, Inter- and intra-annual variations of Pb/Ca ratios in clam shells (Mercenaria mercenaria): a record of anthropogenic lead pollution: Marine Pollution Bulletin, v. 50, p. 1530-1540.

Gillikin, D.P. *et al*., 2006, Shell carbon isotopic composition of *Mytilus edulis* shells: relation to metabolism, salinity, $\delta^{13}C_{DIC}$ and phytoplankton: Organic Geochemistry, v. 37, p. 1371-1382.

Gillikin, D.P. *et al*., 2007, A large metabolic carbon contribution to the $\delta^{13}C$ record in marine aragonite bivalve shells: Geochimica et Cosmochimica Acta, v. 71, p. 2936-2946.

Hansen, J.A. *et al*., 2009, Nitrogen isotopes in mollusks as a proxy for marine productivity: Geological Society of America Abstracts with Programs, v. 41, no.7, p. 475 .

Howarth, 2008, Coastal nitrogen pollution: A review of sources and trends globally and Regionally: Harmful Algae, v. 8, no. 1, p. 14-20.

Huyer, A. *et al*., 1987, Coastal Upwelling off Peru during normal and El Niño times, 1981-1984: Journal of Geophysical Research Oceans, DOI 92:14297-14307.

Jones, D. S. and Quitmyer, I.R., 1996, Marking time with bivalve shells: oxygen isotopes and season of annual increment formation: Palaios, v. 11, p. 340–346.

Kirby, M.X. *et al*., 1998, Stable isotope sclerochronology of Pleistocene and recent oyster shells (*Crossostrea virginica*): Palaios, v. 13., no. 6, p. 560-569.

Kovacs, C.J. *et al*., 2010, *Crassostrea virginica* shells record local variation in wastewater inputs to a coastal estuary: Aquatic Biology, v. 9, p. 77-84.

Kraeuter, J.N., and M. Castagna, 2001 Biology of the Hard Clam: London, Academic Press, 751 p.

Marin, F., and Luquet, G., 2004, Molluscan shell proteins: Comptes Rendus Palevol, v. 3, p. 469-492.

McClelland J.W. *et al*., 1997, Nitrogen-stable isotope signatures in estuarine food webs: a record of increasing urbanization in coastal watersheds: Limnology and Oceanography, v. 42, p. 930-937.

O' Donnell, T.H *et al.*, 2003, Analysis of $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ in organic matter from the biominerals of modern and fossil *Mercenaria spp*: Organic Geochemistry, v. 34, p. 165-183.

Post, D., 2002, Using stable isotopes to estimate trophic position: models, methods, and assumptions: Ecology, v. 83, no. 3, p. 703-718.

Pouget, E.M. *et al*., 2000, The initial stages of template-controlled $CaCO_3$ formation revealed by Cryo-TEM: Science, v. 323, p. 1455-1458.

Risk, M.J., Sayer, B.G., Tevesz, M.J., and Karr, C.D., 1996, Comparison of the organic matrix of fossil and recent bivalve shells: Lethaia, v. 29, no. 2, p. 197-202.

Rowell, K. *et al*., 2010, Nitrogen isotopes in otoliths reconstruct ancient trophic position: Environmental Biology of Fishes, DOI 10.1007/s10641-010-9687-9.

Stapp, P. *et al*., 1999, Stable isotopes reveal strong marine and El Niño effects on island food webs: Letters to Nature, v. 401, p. 467-469.

Stenzel, H.B., 1963, Aragonite and calcite as constituents of adult oyster shells: Science, v. 142, p. 232–233.

Surge, D, *et al*., 2001, Controls on isotopic chemistry of the American oyster, Crassostrea virginica: implications for growth patterns: Palaeogeography, Palaeoclimatology, Palaeoecology, v. 172, p. 283-296.

Surge, D, K. and Walker, K.J., 2006, Geochemical variation in microstructural shell layers of the southern quahog (*Mercenaria campechiensis*): Implications for reconstructing seasonality: Palaeogeography, Palaeoclimatology, Palaeoecology, v. 237, p. 182–190.

Surge, D. *et al*., 2007, Isotope sclerochronology of *Mercenaria mercenaria, M. campechiensis,* and their natural hybrid form: does genotype matter?: Palaios, v. 23, p. 559-565.

Thompson, V. D. and Andrus, C.F.T., in press, Evaluating mobility, monumentality, and feasting at the Sapelo Island Shell Ring Complex: American Antiquity.

Vander Putten E. *et al*., 2000, High resolution distribution of trace elements in the calcite shell layer of modern Mytilus edulis: Environmental and biological controls: Geochimica et Cosmochimica Acta, v. 64, p. 997-1011.

Versteegh, E.A.A., Gillikin, D.P., and Dehairs, F., 2011, Analysis of $\delta^{15}N$ values in mollusk shell organic matrix by elemental analysis/isotope ratio mass spectrometry without acidification: and evaluation and effects of long-term preservation: Rapid Communications in Mass Spectrometry, v. 25, no. 5, p. 675-680

Wanamaker, Alan D. Jr. *et al*., 2006, An aquaculture-based method for calibrated bivalve isotope paleothermometry: Geochemistry Geophysics Geosystems, v. 7, no. 9, doi:10.1029/2005GC001189.

Wanamaker, Alan D. Jr. *et al*., 2007, Experimental determination of salinity, temperature, growth, and metabolic effects on shell isotope chemistry of *Mytilus edulis* collected from Maine and Greenland: Paleoceanography, v. 22, doi:10.1029/2006PA001352.

Watanabe, S. *et al*., 2009, Nitrogen stable isotope ratio in the manila clam, *Ruditapes philippinarum*, reflects eutrophication levels in tidal flats: Marine Pollution Bulletin, v. 58, p. 1447-1453.

Weiner, S., and Hood, L., 1975, Soluble protein of the organic matrix of mollusk shells: a potential template for shell formation: Science, v. 190, no. 4218, p. 987-989.

Weiss, E.T., *et al*., 2002, The effect of nitrogen loading on the growth rates of quahogs (*Mercenaria mercenaria*) and soft-shell clams (*Mya arenaria*) through changes in food supply: Aquaculture. v. 211, p. 275-289.

## Appendix

Table 1A. Summary of $\delta^{15}N$, %N, and sample mass data for all species and standards presented in order of analysis.

| Sample I.D. | Method | Sample Mass (mg) | $\delta^{15}N$ (Air)(‰) | %N |
|---|---|---|---|---|
| B2155 Standard | Untreated | 1.32 | 5.96 | 13.36 |
| B2155 Standard | Untreated | 1.07 | 5.94 | 13.82 |
| B2155 Standard | Untreated | 1.38 | 5.87 | 13.63 |
| B2155 Standard | Untreated | 1.46 | 5.96 | 13.65 |
| B2155 Standard | Untreated | 1.42 | 5.99 | 13.70 |
| B2155 Standard | Untreated | 1.29 | 5.92 | 13.68 |
| B2155 Standard | Untreated | 1.10 | 5.91 | 13.64 |
| Blank Filter | Untreated | N/A | Below 50mV | Below 50mV |
| Acetinalide Standard | Untreated | 0.91 | -1.22 | 9.94 |
| *Mercenaria* 1 | Pretreated | N/A | 8.16 | N/A |
| *Mercenaria* 2 | Pretreated | N/A | 7.38 | N/A |
| *Mercenaria* 3 | Pretreated | N/A | 7.77 | N/A |
| *Mercenaria* 4 | Pretreated | N/A | 6.63 | N/A |
| *Mercenaria* 7 | Pretreated | N/A | 7.68 | N/A |
| Acetinalide Standard | Untreated | 1.01 | 1.76 | 10.23 |
| *Mercenaria* 8 | Pretreated | N/A | 7.70 | N/A |
| *Mercenaria* 9 | Pretreated | N/A | 9.03 | N/A |
| *Mercenaria* 10 | Pretreated | N/A | 7.81 | N/A |
| *Mercenaria* 12 | Pretreated | N/A | 7.91 | N/A |
| *Mercenaria* 13 | Pretreated | N/A | 6.66 | N/A |
| *Mercenaria* 6 | Pretreated | N/A | 8.36 | N/A |
| *Mercenaria* 11 | Pretreated | N/A | 7.58 | N/A |
| *Mercenaria* 14 | Pretreated | N/A | 8.44 | N/A |
| *Mercenaria* 5 | Pretreated | N/A | 7.72 | N/A |
| Acetinalide Standard | Untreated | 1.08 | 1.47 | 10.15 |
| *Mercenaria* 15 | Pretreated | N/A | 8.70 | N/A |
| Blank Filter | Untreated | N/A | Below 50mV | Below 50mV |
| *C. virginica* 2 | Pretreated | N/A | 5.1 | N/A |

| | | | | |
|---|---|---|---|---|
| *C. virginica* 5 | Pretreated | N/A | 8.7 | N/A |
| *C. virginica* 8 | Pretreated | N/A | 8.63 | N/A |
| *C. virginica* 6 | Pretreated | N/A | 9.01 | N/A |
| *C. virginica* 1 | Pretreated | N/A | 5.41 | N/A |
| *C. virginica* 4 | Pretreated | N/A | 6.95 | N/A |
| *C. virginica* 3 | Pretreated | N/A | 6.68 | N/A |
| *C. virginica* 7 | Pretreated | N/A | 8.97 | N/A |
| Blank Filter | Untreated | N/A | Below 50mV | Below 50mV |
| *Mercenaria* 16 | Pretreated | N/A | Lost | N/A |
| *Mercenaria* 17 | Pretreated | N/A | 5.42 | N/A |
| *Mercenaria* 18 | Pretreated | N/A | 9.13 | N/A |
| *Mercenaria* 19 | Pretreated | N/A | 8.64 | N/A |
| *Mercenaria* 20 | Pretreated | N/A | 9.72 | N/A |
| *Mercenaria* 21 | Pretreated | N/A | 9.18 | N/A |
| New Run | | | | |
| B2151 Standard | Untreated | 21.74 | 4.43 | 0.54 |
| B2151 Standard | Untreated | 25.58 | 4.38 | 0.48 |
| B2151 Standard | Untreated | 31.19 | 4.31 | 0.49 |
| B2151 Standard | Untreated | 24.44 | 4.44 | 0.51 |
| B2151 Standard | Untreated | 24.67 | 4.54 | 0.51 |
| B2151 Standard | Untreated | 6.79 | 4.34 | 0.43 |
| B2151 Standard | Untreated | 18.41 | 4.3 | 0.5 |
| B2151 Standard | Untreated | 11.79 | 4.37 | 0.48 |
| B2151 Standard | Untreated | 4.34 | 4.46 | 0.44 |
| Blank | Untreated | N/A | Below 50mV | Below 50mV |
| *Mercenaria* 1 | Untreated | 34.60 | 6.91 | 0.04 |
| *Mercenaria* 2 | Untreated | 34.01 | 6.17 | 0.04 |
| *Mercenaria* 3 | Untreated | 34.11 | 7.32 | 0.05 |
| *Mercenaria* 4 | Untreated | 34.52 | 5.98 | 0.11 |
| *Mercenaria* 5 | Untreated | 34.13 | 7.54 | 0.06 |
| *Mercenaria* 6 | Untreated | 34.16 | 7.61 | 0.07 |
| *Mercenaria* 7 | Untreated | 34.08 | 7.56 | 0.1 |
| *Mercenaria* 8 | Untreated | 34.24 | 7.86 | 0.06 |
| *Mercenaria* 9 | Untreated | 34.86 | 8.53 | 0.07 |
| *Mercenaria* 10 | Untreated | 34.15 | 7 | 0.05 |
| *Mercenaria* 11 | Untreated | 34.36 | 8.74 | 0.07 |
| *Mercenaria* 12 | Untreated | 34.78 | 7.11 | 0.07 |
| *Mercenaria* 13 | Untreated | 33.87 | 6.01 | 0.08 |
| *Mercenaria* 14 | Untreated | 34.44 | 7.22 | 0.04 |
| *Mercenaria* 15 | Untreated | 34.27 | 8.52 | 0.07 |
| Blank | Untreated | N/A | Below 50mV | Below 50mV |

| | | | | |
|---|---|---|---|---|
| Acetinalide Standard | Untreated | 1.13 | 1.32 | 11.03 |
| *C. virginica* 1 | Untreated | 34.75 | 5.49 | 0.24 |
| *C. virginica* 2 | Untreated | 33.86 | 5.46 | 0.29 |
| *C. virginica* 3 | Untreated | 34.09 | 6.61 | 0.27 |
| *C. virginica* 4 | Untreated | 34.54 | 6.66 | 0.26 |
| *C. virginica* 5 | Untreated | 34.17 | 8.38 | 0.33 |
| *C. virginica* 6 | Untreated | 34.18 | 8.75 | 0.31 |
| *C. virginica* 7 | Untreated | 34.88 | 8.9 | 0.27 |
| *C. virginica* 8 | Untreated | 33.55 | 8.52 | 0.25 |
| *Mercenaria* 16 | Untreated | 34.45 | 5.48 | 0.07 |
| *Mercenaria* 17 | Untreated | 34.35 | 4.6 | 0.06 |
| *Mercenaria* 18 | Untreated | 34.27 | 8 | 0.08 |
| *Mercenaria* 19 | Untreated | 34.01 | 8.77 | 0.05 |
| *Mercenaria* 20 | Untreated | 33.18 | 8.52 | 0.06 |
| *Mercenaria* 21 | Untreated | 34.52 | 9.23 | 0.07 |
| Blank | Untreated | N/A | Below 50mV | Below 50mV |
| Acetinalide Standard | Untreated | 0.94 | 1.68 | 10.92 |
| New Run | | | | |
| B2155 Standard | Untreated | 0.94 | 6.01 | 13.5 |
| B2155 Standard | Untreated | 0.99 | 6.01 | 14.11 |
| B2155 Standard | Untreated | 0.84 | 5.96 | 13.75 |
| B2155 Standard | Untreated | 0.87 | 6.07 | 13.82 |
| B2155 Standard | Untreated | 0.99 | 5.91 | 13.75 |
| B2155 Standard | Untreated | 0.84 | 5.93 | 13.91 |
| B2155 Standard | Untreated | 0.95 | 5.88 | 13.65 |
| B2155 Standard | Untreated | 0.86 | 5.79 | 14.15 |
| B2155 Standard | Untreated | 0.47 | 5 | 12.7 |
| B2155 Standard | Untreated | 0.45 | 5.28 | 12.21 |
| B2155 Standard | Untreated | 0.50 | 5.65 | 13.25 |
| B2155 Standard | Untreated | 0.43 | 6.5 | 12.13 |
| B2155 Standard | Untreated | 0.40 | 6.83 | 12.25 |
| B2155 Standard | Untreated | 0.40 | 5.68 | 12.2 |
| Acetinalide Standard | Untreated | 1.39 | 1.6 | 10.63 |
| *M. edulis* 6A | Pretreated | N/A | 9.34 | N/A |
| *M. edulis* 5A | Pretreated | N/A | 9.36 | N/A |
| *M. edulis* 4A | Pretreated | N/A | 9.59 | N/A |
| *M. edulis* 3A | Pretreated | N/A | 9.15 | N/A |
| *M. edulis* 2A | Pretreated | N/A | 9.18 | N/A |
| *M. edulis* 1A | Pretreated | N/A | 9.26 | N/A |

| | | | | |
|---|---|---|---|---|
| *M. edulis* 6C | Pretreated | N/A | 8.77 | N/A |
| *M. edulis* 5C | Pretreated | N/A | 9.51 | N/A |
| *M. edulis* 4C | Pretreated | N/A | 9.12 | N/A |
| *M. edulis* 3C | Pretreated | N/A | 9.1 | N/A |
| *M. edulis* 2C | Pretreated | N/A | Lost | N/A |
| *M. edulis* 1C | Pretreated | N/A | 9.12 | N/A |
| *M. edulis* 6A | Untreated | 35.08 | 8.14 | 0.1 |
| *M. edulis* 5A | Untreated | 35.13 | 9.46 | 0.1 |
| *M. edulis* 4A | Untreated | 34.83 | 7.86 | 0.08 |
| *M. edulis* 3A | Untreated | 35.81 | 8.43 | 0.09 |
| *M. edulis* 2A | Untreated | 33.60 | 8.62 | 0.09 |
| *M. edulis* 1A | Untreated | 34.21 | 8.44 | 0.1 |
| *M. edulis* 6C | Untreated | 35.28 | 8.28 | 0.18 |
| *M. edulis* 5C | Untreated | 33.68 | 8.91 | 0.15 |
| *M. edulis* 4C | Untreated | 34.74 | 8.63 | 0.21 |
| *M. edulis* 3C | Untreated | 34.13 | 8.7 | 0.15 |
| *M. edulis* 2C | Untreated | 34.35 | 8.31 | 0.2 |
| *M. edulis* 1C | Untreated | 34.79 | 8.8 | 0.2 |

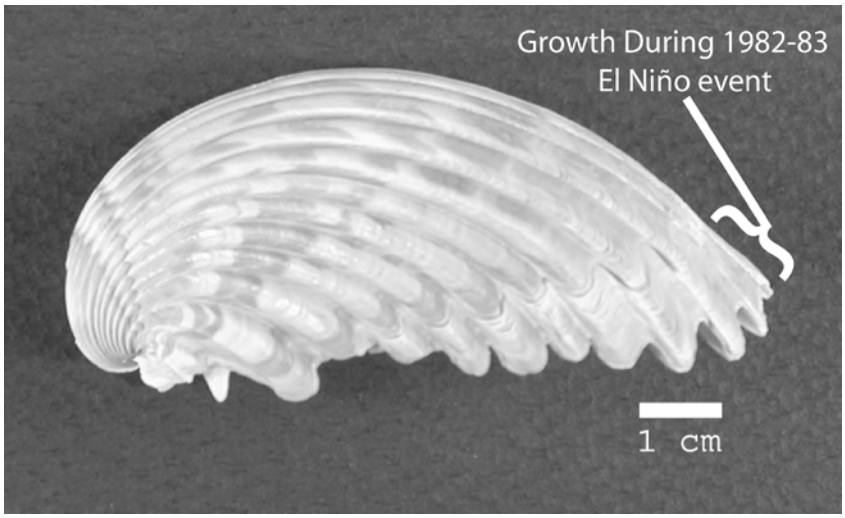


Figure 1A. Profile of *T. procerum* analyzed for $\delta^{13}C$, $\delta^{18}O$, organic content and radiocarbon. Photo from Andrus *et al*., 2005.

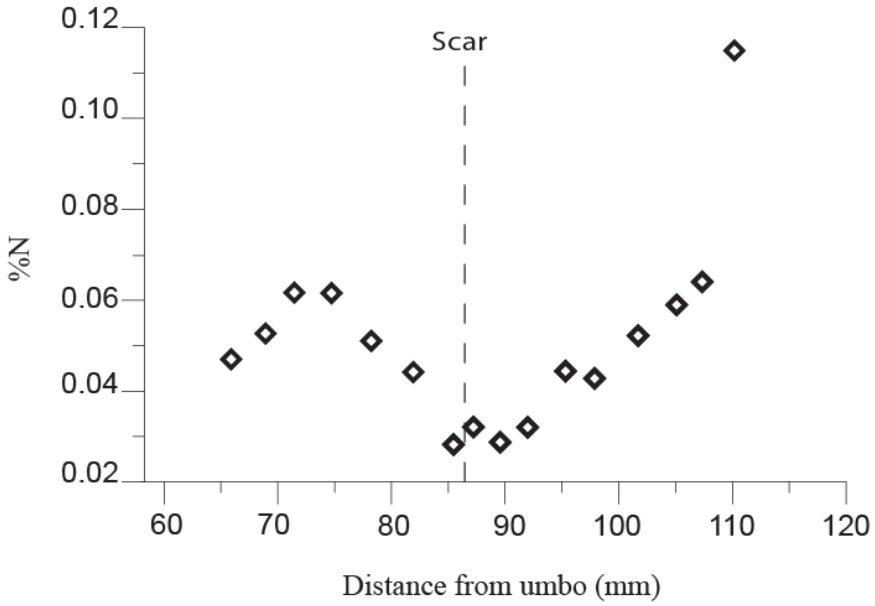


Figure 2A**.** *T. procerum* time series %N data on a shell analogous to the specimen analyzed for organic content. Note steady increase in %N after the onset of El Niño as indicated by the growth scar.

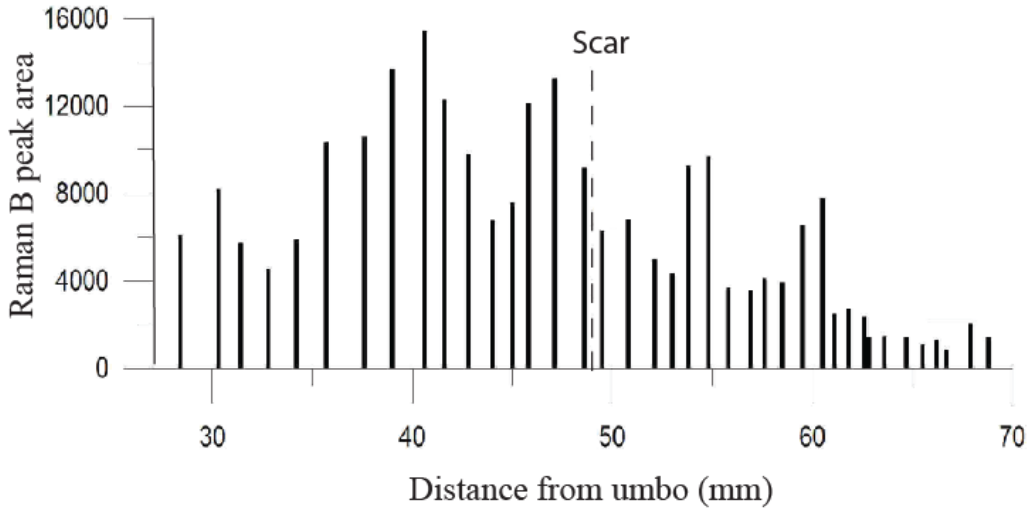


Figure 3A. *T. procerum* time series Raman spectra analysis showing Raman B peak area (organic phase) which correlates to organic content. Note decrease in organic content after the onset of El Niño as indicated by the growth scar. Figure adapted from Etayo-Cadavid, 2010.