Using Polyunsaturated Fatty Acid biomarkers to trace changes in diet of the ribbed mussel, *Guekensia demissa*, in Great Sippewissett Marsh

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Abstract

The abundance and composition of polyunsaturated fatty acids (PUFAs) can be indicators of organic dietary sources and provide insight into metabolic bioprocessing. As PUFAs are acquired through diet, animals can desaturate and chain elongate them during bioprocessing. PUFAs in the ribbed mussel, Guekensia demissa, were quantified and characterized to gain a better understanding of the food web in Great Sippewissett Marsh. I quantified and characterized PUFA’s in the ribbed mussel, Guekensia demissa. Stable isotopes were used to identify the relative proportions of the ribbed mussel’s main dietary sources, phytoplankton shifting from near equal 53/47% phtoplankton/Spartina split near the creek inlet to 30%/70% phytoplankton/Spartina split in the upper regions of the marsh. The $^{15}$N trend in the mussels is consistent with $^{13}$C showing Spartina has a greater influence on the diet of mussels in the upper marsh. Lipids were extracted in an organic solvent (2:1 chloroform:MeOH), using the Folch extraction method and quantified using a gas chromatograph Mass Spectrometer (GCMS). Concentrations of fatty acids are constant throughout the marsh while the degree of unsaturation shifts along the creek gradient becoming more saturated moving away from the inlet. PUFAs reflect changes in mussel diet and physiology at different locations in the marsh.

Key words: Polyunsaturated fatty acids (PUFAs), bioprocessing, chain elongation, desaturation, phytoplankton, suspended detritus

Introduction

Bivalves like the ribbed mussel are often the domain suspension feeders in most New England estuaries. Mussels are found in highly productive systems and will filter what is available in the water column as a food source. Mussels have gills on both sides that are folded forming a pair of gills or demibranchs (Barnes 1987). Mussels have four large, wide filtering surfaces called lamellae that are present on the gills. There are two lamellae on each gill (demibranch). The gills of mussels serve as filters and the gill cilia transport particles trapped in mucus from the gills to the mouth (Barnes 1987). Most bivalves with this type of feeding adaptation will feed on phytoplankton and suspended detritus. Food particles as small as 1 micron are filtered from the water that then pass through filaments. Particles are sorted by weight and size. Small, light particles are retained for ingestion and the heavy, bigger particles are brought to the lamellae where they are rejected and discarded (Barnes 1987).

Investigating the relative proportion of various organic matter sources in biogeochemical cycles within estuarine ecosystems is crucial to understanding the potential responses of these systems to human impacts (Alfaro et al., 2006). In traditional studies of food webs, most have
used gut contents and field observations to follow this organic matter through the food web. Gut contents were examined in a study conducted by Bizerril (1996), where they tracked qualitative changes in the trophic structure in the Sao Joao river fish. The study of estuarine systems food webs presents additional difficulties because estuaries are tropically complex; they support a large variety of primary producers and have a complex detritus based system (Richoux 2008). Stable isotopic composition in animal tissue has been known to correlate with diet ($\delta^{13}$C) and trophic position ($\delta^{15}$N), allowing for scientists to discern the relative importance of specific dietary components. However, the difference between the $\delta^{13}$C in a consumer and what the consumer eats is known to be as large as -1.2 to +4.3 ‰ (Pearson et al., 2003).

Peterson et al. (1985), conducted a study in Great Sippewisset Marsh using multiple stable isotopes ($^{15}$N, $^{13}$C, $^{34}$S) to trace the flow of organic matter. Peterson examined the stable isotopes of the ribbed mussel and its main food sources of plankton and marsh vegetation. Based on bulk isotopes they found that the proportions of diet in the ribbed mussel were dependent on the location of the mussels in the marsh (low, mid, high). The higher in the marsh that the mussels were found the heavier the isotopic signal.

Unlike $\delta^{15}$N and $\delta^{13}$C, fatty acids do not change with the transfer from primary producers to high trophic levels, making fatty acids a suitable biomarker (Alfaro et al., 2006). Fatty acids provide additional information on the type and quality of resources assimilated by organisms over an animal’s life history. Lipid production by primary producers is an important source of nutrition and energy for many invertebrates because they supply polyunsaturated fatty acids (PUFAs) through consumption (Biandolino et al. 2007). Essential fatty acids can not be synthesized and therefore must be acquired through diet, including 20:5ω3 and 22:6ω3 (Richoux, 2008). PUFAs are vital to structural and functional roles in membranes, hence making them essential to the growth and development of organisms (Richoux, 2008). The quantification of PUFA’s is a mechanism that can determine carbon sourcing. PUFAs are long carbon compounds with multiple double bonds and an acid functional group, varying in distribution (Christie, 2003). Recent studies using lipid biomarkers and stable isotopes ($\delta^{13}$C and $\delta^{15}$N) have been able to more accurately identify food sources and the different interactions among trophic levels within estuaries (Peterson et al., 1985; Alfaro et al., 2006). Although, estuaries serve as nurseries for commercially important animals and are a vital connection between fresh and
saltwater little is about the fatty acid composition of many of the organisms in estuarine ecosystems throughout the world (Richoux, 2008).

Using the Peterson et al. (1985) study as a foundation, this study sets out to explore PUFAs and stable isotopes of $^{15}$N and $^{13}$C in the ribbed mussel, *Guekensia demissa*, and its primary food sources, plankton and suspended plant detritus. This study quantified and characterized polyunsaturated fatty acid (PUFA) composition in the ribbed mussel, *Guekensia demissa*. A combination of both stable isotopes and the lipid extraction techniques has proven to be the most effective tool for determining trophic interactions within the complex food webs of estuarine ecosystems (Alfaro et al., 2006). The PUFA biomarkers in conjunction with stable isotopes will be used to quantify the relative proportions of the mussels’ two primary food sources, plant detritus and phytoplankton. This study will lead to a better understanding of the food web trophic structure in Great Sippewissett Marsh and indicate the mussel diet can vary depending upon the availability of food sources and location within Great Sippewissett Marsh.

**Methods**

*Collection*

I collected mussels throughout the (low, mid, high) zones in the Great Sippewissett Marsh, recording the distance in the marsh from Buzzards Bay (fig. 1). Five individual mussels were pooled at five primary sites (fig. 1). Tanner Cunningham collected upland plants to be analyzed for CN (stable isotopic ratios) as part of his Semester in Environmental Science study. I collected detritus samples at two defined sites along the creek channel, during an ebbing tide going into Buzzards Bay (fig. 1). The detritus consisted of a composite of sources (benthic algae, grasses, etc) and I used a 4 liter polyethylene (PE) carboy to collect the sample which was pre-filtered it in the field using a 53 micron Nitex screen to exclude larger particles and zooplankton unavailable to the filter feeding mussels. Mussels sites and detritus sites were analyzed for bulk carbon and nitrogen (abundance and isotopes). Detritus sites were pooled for organic extraction to capture a composite of the marsh, but for the bulk analysis the sites were analyzed separately. I collected phytoplankton during a flooding tide coming in from Buzzards Bay with a PE carboy to collect water samples at the mouth of the channel. I pre-filtered the phytoplankton water samples using a 53 micron screen to filter out
large particulates and zooplankton. I preserved all samples on ice to prevent PUFA degradation in the field.

**Laboratory**

On the same day as the samples were collected I filtered the phytoplankton and suspended detritus samples onto combusted 47mm GF/F filters, immersed them in 2:1 chloroform : MeOH and stored them at -30°C in a 40 ml vial pending organic extraction. I filtered additional phytoplankton and suspended detritus onto 25mm GF/F filters for bulk CN (carbon and nitrogen) elemental analysis. I rinsed the mussel samples and stored them at -80°C overnight following field collection.

I extracted five samples for organic analysis: 3 mussel sites, 1 phytoplankton, 1 pooled detritus. The other two intermediate mussel sites were analyzed for bulk carbon and nitrogen (abundance and stable isotopes) along with all detritus and phytoplankton sites. The length and width of the mussels from the five pooled sites was measured. I broke the mussel shells with a hammer and placed the entire interior contents in a clean and combusted 40 ml vial, freeze dried for two days, and then homogenized using a coffee grinder. For a subsample, I weighted three of the mussel sites for lipid extraction into combusted 16mm Pyrex test tubes, averaging a subsample weight of 67 mg. In order to quantify my samples on the Gas Chromatograph Mass Spectrometer (GCMS) I added an internal standard: 200µl of standard mixture was added to each of the three mussel samples, which consisted of 510.16 µg/ml 21 FAL (fatty alcohol), 563.10 µg/ml 23 FA (fatty acid), 514.0 µg/ml five alpha cholestane, and 565.40 µg/ml 36 ALK (alkane). I added 20 µl of a more dilute standard to the phytoplankton and detritus samples, which consisted of 362.1 µg/ml 21 FAL, 348.4 µg/ml 23 FA, 354.0 µg/ml 5 α cholestane, and 357.1 µg/ml 36 Alkane.

After standard additions I immersed the samples in 2:1 chloroform : methanol in preparation for ultrasonicatic extraction. I centrifuged the three mussel samples for ten minutes and sonicated (cold recirculating bath, <7°) for 5 mintues. I removed the organic solvent to separatory funnels and the centrifuge/ultrasonic extraction repeated 3x 4ml solvent, combing rinses, 10 ml of 0.88% KCL(aq) was added (1:4 volume) to remove water soluble carbohydrates and proteins from the organic extract into an aqueous layer. To make sure the rest of the organic
layer is obtained I did a back extraction with 2:1 chloroform methanol. I rotovaped each of the five extracts in pear flasks for ten minutes. I dried the extract with sodium sulfate columns and rinsed them with chloroform. The 20% aliquot of the extracts was transesterified with 15% anhydrous methanolic HCl and 85% acetyl chloride. I let this reaction set overnight after flushing the tubes with nitrogen gas in 55°C to break complex molecules into components and transform the free fatty acids into methyl ester derivitatives. I derivitized the extract into a trimethylsilyl (TMS) derivative and analyzed with Agilent technologies 7890A Gas Chromatograph Mass Spectrometer (GCMS).

After looking at the total extract on the GCMS, the totals were separated enough on the GCMS that it was possible to distinguish between different compounds. Using Chrom perfect (2003) 32-bit Chromatography Data system: version 5.5.2, the chromatographs baseline was straightened and the peak areas adjusted to account for the total area the GCMS measured. Additionally, I used Enhanced Chem. Station (2008) E.02.00.493, Agilent Technologies to quantify and identify the compounds present in samples.

The bulk samples were analyzed with the Europa 20-20 CF-IRMS interfaced with the Europa ANCA-SL elemental analyzer. The following mass balance equation is used to determine the percentage of diet in the mussels that comes from phytoplankton and *Spartina alterniflora* material, where x= the percentage of phytoplankton and (1-x)= the percentage of *Spartina alterniflora*: mussel site $\delta^{13}C = \text{phytoplankton} \delta^{13}C \times x + (1-x) \text{Spartina} \delta^{13}C$. The same equation is used to determine the composition of the detritus samples collected: detritus $\delta^{13}C = \text{phytoplankton} \delta^{13}C \times x + (1-x) \text{Spartina} \delta^{13}C$.

**Results**

*Isotopic bulk CN*

The bulk stable isotopes show a significant gradient in the mash, depicting the ribbed mussels diet. The mussel’s $\delta^{13}C$ vary from -18 to -16‰. Between sites one and three, the mussels tend to get lighter (-18—18.6, respectively). Between site four and five the mussels tend to get heavier (-17.1—-16, respectively). The mussels collected closes to the inlet have an
isotopic $\delta^{13}C$ that is close to the phytoplankton $\delta^{13}C$ of -22.1‰ (2012) and 21.3‰ (1985) (fig. 2). Mussels collected further up the creek have an isotopic $\delta^{13}C$ that is close to the Spartina alterniflora, which has a value of -13.4‰ (2012) and -13.1‰ (1985) (fig. 2). The upland plants have a $\delta^{13}C$ of -27.49‰ (fig. 5). Detritus site one has a $\delta^{13}C$ that is close to the $\delta^{13}C$ of phytoplankton, which has a $\delta^{13}C$ of -22.3‰. Detritus site two has a $\delta^{13}C$ that is close to that of Spartina alterniflora, which is -19.1‰. Mussels at site one have an $\delta^{13}C$ isotopic composition that reflects a diet of 53% phytoplankton and 47% Spartina (fig. 2). Mussels in site two and three both show an isotopic composition that reflects a diet of about 60% phytoplankton and 40% Spartina. Mussels in site four reflect a diet of 43% phytoplankton and 57% Spartina. Lastly, mussel site five exhibited a diet of 30% phytoplankton and 70% Spartina (fig. 2).

The $\delta^{15}N$ has a range of 9.1 and 7.6‰ between sites one and five respectively (fig 4). The phytoplankton has a $\delta^{15}N$ of 7.8‰ and the Spartina alterniflora has a $\delta^{15}N$ of 5.8‰. The further from the inlet the lower the $\delta^{15}N$ %. The upland plants have a $\delta^{15}N$ of 1.54‰ (fig. 5). The detritus site one based on the $\delta^{15}N$ is comprised of 100% phytoplankton and detritus site two is comprised of 94% phytoplankton and 6% Spartina.

**Organic extracts**

The major of fatty acids detected in my samples are indicated in table one. The total fatty acid concentrations remain relatively constant at all mussel sites. The phytoplankton and the detritus sites have constant total fatty acid concentrations as well. In $\mu g \, g^{-1}$ carbon mussel site one has a total fatty acid concentration of 78931, mussel site four 74323, and mussel site five 78529. The detritus has a total fatty concentration of 28894 $\mu g \, g^{-1}$ and the phytoplankton have a concentration of 28527 $\mu g \, g^{-1}$ Carbon. There is a distinct difference among the sites between the total polyunsaturated fatty acid (PUFA) concentrations. Mussel site one has a PUFA concentration of 25786 $\mu g \, g^{-1}$ Carbon, mussel site four has a concentration of 20676 $\mu g \, g^{-1}$ Carbon and mussel site five has a concentration of 21418 $\mu g \, g^{-1}$ Carbon.

The individual PUFA compounds within my sample sights show that the mussel sites have a larger percentage of PUFAs that are of a higher saturation than there food sources, detritus and phytoplankton. The mussel sites on average are comprised of 7.4% 22:6, phytoplankton 5% 22:6 and detritus 2.6% 22:6. Additionally, the mussels have a higher
percentage of the longer chained PUFAs than their dietary sources (fig. 6). Most of the phytoplankton and suspended detritus is comprised of 12.6%, 8% 18:4 ω3, respectively. Alternatively, the majority of the mussel sites are comprised of 10% 20:5 ω3 (fig 6).

The individual PUFA concentrations become more saturated when moving up the creek. The 20:4 ω3 and 22:5 (x10) show an increase in concentration in relation to moving up the creek (fig. 7 &8). Mussel site ones’ (200m) concentration of 20:4 ω3 is 3432 µg g⁻¹ C⁻¹ and mussel site three (625m) is 5322 µg g⁻¹ C⁻¹. Mussel site ones’ (200m) concentration of 22:5 (x10) is 3756 µg g⁻¹ C⁻¹ and mussel site three (625m) is 3394 µg g⁻¹ C⁻¹. The 20:5 ω3 and 22:6 ω3 showed a downward trend with the mussel sites increasing in distance from the inlet (fig. 7 & 8). The 20:5 ω3 concentration at mussel site one (200m) is 10080 µg g⁻¹ C⁻¹ and at mussel site five (625m) is 7702 µg g⁻¹ C⁻¹ (fig. 7). The 22:6 ω3 concentration at site one (200m) is 9361 µg g⁻¹ C⁻¹ and at site five (625m) the concentration decreases to 7228 µg g⁻¹ C⁻¹.

Discussion

Peterson’s et al. (1985) isotopic data is consistent with my data. The δ¹³C of our phytoplankton and Spartina alterniflora are within a small margin of error of each other. Both data sets indicate that carbon isotopes in the mussels get heavier along a gradient moving up the marsh reflecting the increase in Spartina in their diet. The mussel sites closest to the inlet have an isotopic δ¹³C that is closest to that of phytoplankton indicating an increased phytoplankton diet. Additionally, my detritus site that is closest to the inlet has a δ¹³C that is closest to that of the phytoplankton signal and the detritus site farther from the inlet has a δ¹³C that is closer to that of Spartina. This elutes that the mussels’ closest to the inlet are filtering detritus that is enriched with phytoplankton as well as filtering phytoplankton directly from the water column. The δ¹⁵N trend in the mussels is consistent with δ¹³C showing Spartina having a greater influence on the diet of mussels in the upper marsh. The mussel sites are δ¹⁵N enriched indicating increased fractionation with increasing trophic levels. Thus, the location of an organism in the marsh can determine the type of food available and the isotopic ratios in its tissues (Peterson et al. 1985). My mixing model δ¹⁵N data indicates that the suspended detritus sites have a prevalent upland
marsh vegetation signal. The detritus site two indicates that upland plants could be the nitrogen source that is making up a proportion of the suspended detritus.

Although, the total concentrations of fatty acids are constant the degree of unsaturation shifts along the creek gradient. This is shown with the downward trend in the 22:6 ω3 and 20:5 ω3 (fig. 7 & 8). Additionally, the 22:5 and 20:5 ω3 are increasing in relation to the mussel sites moving further away from the inlet. According to Biandolino (2007), in mollusks the fatty acid composition is characterized by 20:5 ω3 and 22:6 ω3, where this pattern reflects the nature of the ingest food represented by phytoplankton. The mussels have higher PUFA and fatty acid concentrations than their dietary sources due the bioaccumulation of the compounds. The mussels exhibit higher unsaturation and have greater percentages of longer chained carbon PUFAs than their dietary sources (fig. 6). When the mussels are filtering the phytoplankton and the suspended detritus they are chain elongating and desaturating the PUFA compounds during bioprocessing. Changes throughout the marsh in the concentration of PUFAs (fig. 7& 8) could be related to changes in diet sources or varying physiology of the mussels at different locations in the marsh.

Conclusion

Both my isotopic results as well as the PUFA compounds indicate that the mussels closes to the inlet main food source is phytoplankton and as you move up the creek the mussel’s main diet begins to consist of suspended plan detritus. The relative proportion of each dietary source is determined by marsh location. For further analysis, using a GCirMS for compound specific isotope analysis of the carbon in individual PUFAs could possibility provide insight into the extent of carbon fractionation through biosynthetic processes, due to PUFA chain elongation by mussels.

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References


Figures and tables

Figure 1. Map of field site with specific site locations. Stars are mussel collection sites (Yellow symbols indicate samples to be extracted for organics), Circles are detritus collection sites (will be pooled for organic analysis, run individually for bulk elemental analysis). The square is the phytoplankton collection site.

Figure 2. The percent contribution of each dietary source, Phytoplankton or Spartina alterniflora, at each mussel site, one through five throughout Great Sippewissett Marsh.

Figure 3. $\delta^{13}C$ for mussels, phytoplankton and suspended detritus in relation to the distance in the creek from the Buzzards Bay inlet in Great Sippewissett Marsh for Peterson et al. (1985) represented by the open symbols and my research represented by the colored symbols.

Figure 4. $\delta^{15}N$ for mussels, phytoplankton and suspended detritus in relation to the distance in the creek from the Buzzards Bay inlet in Great Sippewissett Marsh for Peterson et al. (1985) represented by the open symbols and my research represented by the colored symbols.

Figure 5. The mussel transect data showing $\delta^{15}N$ plotted as a function of $\delta^{13}C$.

Table 1. Fatty acid composition ($\mu$g g$^{-1}$ C$^{-1}$) of phytoplankton, suspended detritus, and mussel sites (1, 4 & 5).

Figure 6. Percentage of PUFA’s (18:2, 18:3, 18:4 ω3, 20:4 ω6, 20:5ω3, 22:5, and 22:6ω3) in phytoplankton, suspended detritus and the mussel sites averaged together.

Figure 7. Individual PUFA’s 20:4ω3 and 20:5ω3 concentrations in $\mu$g g$^{-1}$ C$^{-1}$ at mussel sites, phytoplankton, and detritus samples.

Figure 8. Individual PUFA’s 22:5 (multiplied by 10) and 22:6 ω3 concentrations in $\mu$g g$^{-1}$ C$^{-1}$ at mussel sites, phytoplankton, and suspended detritus samples.
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Mussels: $\delta^{13}C$

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<td>47.5</td>
</tr>
<tr>
<td>22:2</td>
<td>0.0</td>
<td>4543.3</td>
<td>4288.3</td>
<td>4566.7</td>
<td>44.0</td>
</tr>
<tr>
<td>22:1</td>
<td>1958.1</td>
<td>0.0</td>
<td>101.9</td>
<td>0.0</td>
<td>2362.3</td>
</tr>
<tr>
<td>22:0</td>
<td>308.1</td>
<td>73.7</td>
<td>32.9</td>
<td>0.0</td>
<td>369.7</td>
</tr>
</tbody>
</table>

Table 1. Fatty acid composition (µg g⁻¹ C⁻¹) of phytoplankton, suspended detritus, and mussel sites (1, 4 & 5).
Figure 6. Percentage of PUFA’s (18:2, 18:3, 18:4 ω3, 20:4 ω6, 20:5ω3, 22:5, and 22:6ω3) in phytoplankton, suspended detritus and the mussel sites averaged together.
Figure 7. Individual PUFA’s 20:4ω3 and 20:5ω3 concentrations in µg g⁻¹ C⁻¹ at mussel sites, phytoplankton, and detritus samples.
Figure 8. Individual PUFA’s 22:5 (multiplied by 10) and 22:6 ω3 concentrations in µg g$^{-1}$ C$^{-1}$ at mussel sites, phytoplankton, and suspended detritus samples.