Characterizing the molecular composition of epicuticular waxes of vegetation and in surface sediments in Great Sippewissett marsh, Falmouth MA

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Abstract:

Epicuticular plant waxes are nearly omnipresent in the natural environment and carry with them molecular and isotopic information specific to their species of origin. These waxes can provide that information in real time when collected in aerosols, or from the distant past when extracted from ancient sediments. In order to better constrain the relationship between an ecosystems plant community and the molecular wax signal being recorded in its sediments, vegetation and surface sediment samples were collected from a Massachusetts salt marsh and analyzed to determine the abundance and chain length distributions of n-alkanes, n-fatty alcohols, and n-fatty acids. This molecular “fingerprinting” yielded both qualitative and quantitative evidence that the species diversity and relative ratio of C4 to C3 plant biomass in an ecosystem can be recorded in surface sediments by plant waxes. This type of analysis, in conjunction with compound specific isotopic determination may serve to enhance the capabilities of epicuticular plant waxes as a paleo-environmental proxy.

Introduction:

Much of the existing knowledge of our planets past climate and ecosystems has been extracted from the soils and bedrock beneath our feet. There are many methods to obtain this information, from the exhumation of plant and animal fossils to the chemical analysis of sediment cores. Stable isotope geochemistry has been vital in the exploration of these natural records. Isotopic ratios of oxygen, carbon and hydrogen are preserved by the interplay between biotic and abiotic processes, and allow us to reconstruct dynamic images of the paleo-environment.
The accuracy of climate models today is strongly dependent on our understanding of the carbon cycle and how atmospheric CO₂ levels have changed through time. Stable carbon isotopes can be used to better understand how the biosphere changes and is changed by variance in carbon dioxide levels and climate. The ratio between $^{13}$C and $^{12}$C in plant tissues can provide insight into these changes. It varies as a result of atmospheric ratios, differences in plant photosynthetic pathway, and stress inducing climatic variation. However, it is logistically difficult to sample whole ecosystems for this kind of data. Plant waxes can provide us with a biomarker of this activity and allow us to extract information from a select few places as opposed to thousands.

The cuticle, a waxy, protective covering produced by leaf epithelial cells, serves many functions including physical protection from abrasives and dirt, internalization of gas exchange surfaces (allowing transpiration control), and shielding from bacterial and fungal pathogens. The surface of the cuticle is coated with a thin layer of wax that serves as an additional layer of protection. These epicuticular waxes are constantly being ablated and are nearly omnipresent in the natural environment in the form of micrometer scale crystals. Figure 1 shows a few micrographs of these crystals (Koch 2008). Their variety of physical structure is an indication of their molecular diversity. This diversity is largely contained within three main compound classes, n-alkanes, n-alcohols, and n-acids.

Wind and water mix waxes during transport and samples can contain the integrated signal from whole continents, in which case they can be used to model terrestrial carbon uptake in near real-time (Conte and Weber 2002). This is because the carbon bearing waxes accurately record bulk plant carbon fractionation (Conte and Weber 2003). This allows for a small sample size to represent a huge ecosystem. Plant waxes are also valuable for their recalcitrance, and their often
unique molecular signature. In sediments, plant tissues and byproducts are represented in soil organic carbon and record the isotopic signature of the parent material. As the material is respired away, pedogenic carbonates form that may persist over long time scales. However, this relationship is not well constrained and factors including soil respiration and atmospheric diffusion may alter $\delta^{13}C$ values (Stevenson 2005). An alternative to bulk soil carbon analysis may lie in the use of compound specific isotope ratios of plant wax compounds. While bulk plant material may decompose quickly, sometimes being altered in its isotopic composition therein (Ember et al 1987), plant waxes have been extracted and analyzed from 9 million year old sediments to yield a continuous record of climatic variation (Feakins et al 2005).

Within a given ecosystem, waxes primarily enter the sediment record alongside other bits of detrital plant matter (also wax containing) and contribute to a highly refractory pool of waxes (figure 2). This study focused on the collection and analysis of marsh vegetation and sediment samples that may help to characterize the relationship between local plant diversity and the epicuticular wax signal concurrently recorded in marsh surface sediments. Detailed fingerprints of the waxes of different plant species were taken to help to add an additional layer of source information to knowledge obtained from compound specific isotope studies. Carbon stable isotopes were also measured to complement the wax analysis, providing information on average autumn isotopic composition for a diversity of salt marsh plant species.

**Methods:**

All field work was completed in one day at Great Sippewissett salt marsh in Falmouth, Massachusetts. The whole of the marsh measures approximately 0.5 km in width by 1.5 km in length. Marsh platforms are largely flat with high moisture, high organic content sediments (peat) and dominated by *Spartina alterniflora* (C4, creek bank/low marsh) and *Spartina patens*
Areas of drier sandy hummocks exist in the southern portion of the marsh characterized by the presence of *Ammophila* (C3 dune grass), *Iva* (C3 deciduous shrub), and *Chamaecyparis* (C3 evergreen). Areas of marsh fringe to the east are typically quite dense and shrubby, with the presence of all plants found in the hummocky areas as well as *Baccharis* (C3 shrub) and *Myrica* (C3 shrub), with some *Phragmites* (C3 reed) present in the wetter bordering regions proximal to the bike path that cuts northwest across the eastern marsh edge. All aforementioned plant species were collected, eight in total. Leaf clippings were made with scissors, with three leaves being clipped from two individuals at four different locations (different for each species) across the marsh to achieve spatially homogenized samples. Four different surface sediment samples were collected, spanning the west (ocean side) to east (land side) of the estuary (figure 3). These samples were taken with a garden trowel from open marsh pans (figure 4), where the long side edge would be inserted a few centimeters into the soil and drawn laterally to produce an often cohesive “pancake” of surface sediment. All samples, sediment and vegetation, were placed in clean plastic bags for transport to the lab. There, all samples were freeze dried and ground finely in order to homogenize and prepare for analysis. Roughly 2 mg of soil and 4 mg plant material were weighed out, sediment samples were acidified, and all were sent to the MBL isotope lab for bulk carbon analysis. A subset of these samples was selected for wax extraction. Both *Spartina* species, *Ammophila*, and *Baccharis* were chosen to represent the marsh dominant and marsh fringing species present today. Around 500 mg of each plant sample was weighed out. A quantitatively pooled subsample from the four surface sediment collections was made, with equal contributions totaling 500 mg. All samples were placed in solvent rinsed 16 mm Pyrex tubes with a screw cap outfitted with a Teflon liner. 200 μl of internal standard was added here in order to provide the basis for quantifying wax
compounds after extraction. This standard contained 510.16 μg ml⁻¹ of 21 fatty alcohol, 514.00 μg ml⁻¹ of 5α cholestane, 563.10 μg ml⁻¹ of 23 fatty acid, and 565.40 μg ml⁻¹ of 36 alkane.

Samples were extracted by ultra-sonication in cold (-5°C) methylene chloride for five minutes, with two subsequent 4 ml methylene chloride rinses. All extract was pipetted into fritted glass funnels over separatory funnels. After a Folch extraction, extract was filtered through anhydrous sodium sulfate to remove excess H₂O. A 20% aliquot of each total lipid extract was transesterified with 2ml methanolic hcl incubated at 55°C. After re-extraction into methylene chloride, samples were evaporated with nitrogen and re-suspended in 50 μl of pyridine. 50 μl of BSTFA was added to the sample before a single hour incubation at 55° C to create volatile trimethylsilyl derivatives in preparation for GCMS analysis. Samples were again evaporated with nitrogen gas then finally re-suspended in methylene chloride. An Agilent GCMS with 60m of 0.25 μm polyamide coated capillary column was used to analyze the samples. 1 μl of each sample was injected. The temperature program began with 2 minutes at 50°C, ramped at 10° min⁻¹ to 150°, then at 4° min⁻¹ to 320° where it remained for 20 minutes. Quantification and identification of n-alkanes, n-fatty alcohols, and n-fatty acids was completed on two computer softwares, Agilent Enhanced Data Analysis and Justice Laboratory Software’s ChromPerfect.

Three different mixing models were employed in calculating relative vegetative input to marsh surface sediments. A mixing calculation based on the bulk isotopic analysis of the four different sediment samples was performed in order to obtain the signature of the homogenized sample. It followed the formula:

\[
\delta^{13}C_{ss} = (\%C_1 * M_1 * M_{1234}^{-1} * \delta^{13}C) + (\%C_2 * M_2 * M_{1234}^{-1} * \delta^{13}C) + (\%C_3 * M_3 * M_{1234}^{-1} * \delta^{13}C)
+ (\%C_4 * M_4 * M_{1234}^{-1} * \delta^{13}C)
\]
Where $\delta^{13}C_{ss}$ is the carbon signature of the homogenized surface sediment sample, $M_{1-4}$ are the contributing masses of the sample sites 1-4 to the mixture, and $M_{1234}$ is the total mass of the homogenized sample. $\%C_{1-4}$ are the carbon percentages of each sample as found in table 1. $\delta^{13}C_{ss}$, the average $\delta^{13}C$ of Spartina, and the average $\delta^{13}C$ of the C3 plants in table 1, were then used to determine the relative inputs of C4 and C3 plant matter to surface sediments using a basic two end member mixing model of the form:

$$\delta^{13}C_{ss} = x (\delta^{13}C_{C3}) + (1-x)(\delta^{13}C_{C4})$$

To attempt to quantify the relative inputs of characterized plant waxes to the surface sediments, a multivariate mixing model software, IsoSource version 1.3.1, available on the EPA’s website was used. Here, the three main plant wax compound classes, alkanes, alcohols, and acids were treated as isotopes and their input values were the relative class abundances within a sample. Values from the four analyzed plant species were input as sources, with values of the homogenized sediment sample as the target mixture. A range of feasible species contributions by % weight was provided. In order to adjust from inputs by wax weight to % biomass of plant species, the relative species contribution percents were divided by a ratio of the plant wax concentration, and renormalized to 100% to give an estimate of plant species presence by biomass. Lastly, a sum of squared differences model was used to calculate relative species presence using relative abundance values of 6 n-alcohols (22,24,26,28,30) and 6 n-acids (24,26,28,30,32,34). N-alkane distributions were excluded from this calculation due to the erroneously small percentage of 29 alkane in sediments.

Results:
Bulk carbon isotopic analysis (table 1) yielded a range of 37% to 49% carbon amongst the eight vegetation samples, while the four soils samples ranged from 4% to 25%. The *Spartina* grasses were the only C4 specimens in the analysis, and had $\delta^{13}C$ values of -13.4‰ and -13.6‰ for *S. alterniflora* and *S. patens* respectively. The C3 dune grass *Ammophila* was -25.9‰ and the C3 shrub *Baccharis* was -27.1‰. Other species analyzed, all C3, ranged from -25.6‰ to -29.7‰. Surface sediment samples varied between -15.4‰ and -20.8‰ while the suspended waterborne detritus samples were -22.3‰ and -19.1‰ respectively. Total combined alkane (24-31), alcohol (22-32), and acid(24-34) concentrations varied in all four vegetation samples. While this variance was small amongst the *Spartina* grasses and *Baccharis* shrub, with values around 2000 μg gram dry weight (gdw)$^{-1}$, the dune grass *Ammophila* had over 7000 μg gdw$^{-1}$.

Concentration in the homogenized sediment sample was one order of magnitude lower at 280 μg gdw$^{-1}$ (figure 5). Relative wax compound class abundances (figure 6) for the *Spartina* grasses were similar and showed an alcohol dominance (both 44%). These distributions were relatively even in comparison to *Ammophila* and *Baccharis*, which both had a much higher alcohol abundance, 76% and 73% respectively. Within class n-alkane distributions showed both *Spartina* grasses, *Ammophila*, and the waterborne detritus with a bell curve shape peaking at the 29 carbon chain length. *Baccharis* also had a bell shape, though peaking at 27, while the sediment sample had a bimodal distribution with peaks at 25 and 31(figure 7). Within class n-alcohol distributions were the most diverse of the three compound classes. *Ammophila* was 91% composed of chain length 26, while both *Spartina* grasses had a very dominant 32 length presence. *Baccharis* was more normal in its distribution, with a peak at 28. Both sediment and detritus samples showed a distribution peaking at 32 like the *Spartina* grasses, though with a higher level across all other chain lengths(figure 8). In the n-acid class, both *Ammophila* and *Spartina* had the largest peak at
30, with *Ammophila* bell shaped and *Spartina* bimodal with the other peak at 24. *Baccharis* also exhibited a somewhat bimodal shape, with peaks at 24 and 28. Sediment and detritus samples showed bimodal distributions peaking at 24 and 30 like *Spartina*, with the detritus sample having lower “amplitude” in its shape (figure 9). Results from the quantitative mixing model are shown in table 2.

**Discussion:**

The substantial discrepancy between wax concentration in *Ammophila* and other genera was an interesting result that has implications for interpreting integrated wax signals. The driver of this large increase in wax compound concentration (around 3x that of the other species) is likely climatic. It seems logical that plant species exposed to more environmental stress like physical abrasion or drought may benefit from increased wax concentrations. Indeed, studies have shown that plants within a species are capable of increasing epicuticular wax concentrations during times of water stress and decreasing them during periods of low light (Upadhyaya 1994). If one plant species has significantly greater epicuticular wax concentrations, it is very possible that it will be over represented in terms of species presence in the sediment record. Model calculations of biomass had to take this into account.

In a qualitative sense, it is apparent from the overall and within class abundances that marsh surface sediments are indeed integrating a plant wax signal from multiple plant species present in the marsh. Relative class abundances of n-alkanes and n-alcohols are well within the bounds of the four plant species characterized, though a much higher percentage of n-acids suggests the presence of an acid rich contributor that was not characterized in this study. This unknown must also have a relatively greater input to the waters of the marsh creek, which is in fact dominated by the acid class. Resemblances of within class distributions are variable. Sediment wax alkane distributions show a presence of chain length 25 which is proportionally higher than in
any of the vegetation samples. It is important to note that the low level of 29 alkane in the sediment sample is a result of poor chromatographic resolution between 29 alkane and the added 5 alpha cholestane internal standard. The natural odd dominance of alkanes dictates that 29 should be more abundant than 28, which it is not. This issue did not arise with the detritus sample which has a distribution quite similar to *Spartina*, though with a relative increase in 27 alkane that suggests inputs from the more 27 enriched *Ammophila* and *Baccharis*. It is also worth noting that alkane distributions for all grasses were quite similar, despite differences in photosynthetic pathway Alcohol distributions are the most unique between plant genera, and the relation of these signatures to that found in the sediment and detritus is striking. The signatures of the depositional environments are similar in their overall shape to the *Spartina* grasses. However, it is impossible for the sediments and detritus to have relatively elevated lower chain length alcohols without the additional presence of wax compounds from *Ammophila* and *Baccharis*. The n-alcohols provided the most distinctive wax molecular signatures and thus allow for an easier characterization of the integrated signal. The n-acid distributions of the sediments and detritus again look much like that of the *Spartina* grasses, though with the highest abundance in the 24 acid instead of 30 as in *Spartina*, suggesting inputs from a more short chain dominant source like *Baccharis*.

Three mixing models were employed in an illustrative attempt to quantify the inputs of the four plant species to the surface sediments and their overall presence in the marsh. Bulk carbon, IsoSource (between class) and excel based sum of squared differences (SSD)(within class) techniques are presented. Table 2 lists the results from these various models. These models were run for the waterborne detritus but had substantial enough inaccuracy to exclude on the grounds of a missing fatty acid rich source. It is important to note that all % values are of marsh biomass except the ranges colored in blue which represent the actual wax presence in the sediment by weight. All sediment models predict a substantial dominance of *Spartina patens* grass, 62-71% in wax presence and 68 to 79% in biomass. Surprising is how low the modeled abundance of *Spartina alterniflora*
is, with estimates ranging from 0 to 2% for biomass and 0-7% for wax abundance. Models based on relative class abundance and within class chain length abundances produced predictions of a *Spartina* (C4) dominated marsh with 20-30% C3 biomass.

The most striking results are the exact agreement between the relative C3/C4 biomass inputs of the bulk carbon calculation and the within class distribution calculation. Interestingly, this suggests the waxes in the surface sediments collected were deposited along with significant amounts of plant material, and not by the aeolian transport of epicuticular wax particles alone. An extraction of a sediment with a very *Spartina* indicative bulk signature (around -13.5‰, or perhaps like sediment 2 at -15.4) could provide insight into whether sediments may actually be picking up a signal from ablated wax particles alone. This is a relationship that will be important to constrain. If few compounds are deposited in the form of lone epicuticular wax in marsh sediments than wax molecular and isotopic mixing models of ancient sediments may be good proxies for the bulk content that has long decomposed or changed in its composition. It is however, very possible that these wax compounds are deposited from aerosols and periodic flooding in such a way that distal sources could be represented by molecular and compound specific analyses, but not at all in bulk carbon analyses. It is also worth noting that while there seemed to be some agreement amongst model results; it is very good that there are discrepancies and holes. It would be worrisome to see models flawlessly mimic sediment samples based on only four sources. It makes room for the expansion of the method, as there can be more and more variables added with what is hopefully an increase in accuracy. What is important now is to conduct more extensive wax molecular assays of vegetation and sediment samples, as well as an accurate plant biomass survey of the surrounding areas. This would help to frame the predictions of the molecular results within quantitative measurements of species stock and diversity in the ecosystem. This diversity information could provide very crucial insight as to the identity of end members in compound specific isotopic
analysis models. They could even help to frame shifts in isotopic signature of a non-varying population. where shifts may be a direct result of environmental stress (Stevenson 2005) and resulting decrease in fractionation, not of a change between proportion of C4 and C3 species. Plant wax biomarkers in sediments show an enormous potential for paleoclimatology and paleoecology studies and will benefit greatly by the resolution provided by further study and molecular characterizations.
References


Figure 1: epicuticular wax crystals of multiple plant species
(Koch 2008)
Figure 2: Schematic of plant wax deposition in a marsh environment
Figure 3: Orthographic map of Great Sippewissett marsh, and the location of the surface sediment and suspended detritus collection sites
Figure 4: Photographs of sediment collection locations, trowel indicates location of sample where present
Table 1: Results from the bulk carbon isotopic analysis. The plant species colored in dark green were those selected for the wax analysis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Description</th>
<th>%C</th>
<th>δ13C (‰ vs. PDB)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ammophila breviligata</em></td>
<td>C3, dune grass</td>
<td>42</td>
<td>-25.9</td>
</tr>
<tr>
<td><em>Spartina alternifora</em></td>
<td>C4, marsh grass</td>
<td>39</td>
<td>-13.4</td>
</tr>
<tr>
<td><em>Spartina patens</em></td>
<td>C4, marsh grass</td>
<td>43</td>
<td>-13.6</td>
</tr>
<tr>
<td><em>Baccharis halimifolia</em></td>
<td>C3, marsh fringe shrub</td>
<td>46</td>
<td>-27.1</td>
</tr>
<tr>
<td><em>Phragmites australis</em></td>
<td>C3, reed</td>
<td>44</td>
<td>-27.2</td>
</tr>
<tr>
<td><em>Iva frutescens</em></td>
<td>C3, shrub</td>
<td>37</td>
<td>-29.7</td>
</tr>
<tr>
<td><em>Chamaecyparis thyoides</em></td>
<td>C3, &quot;white cedar&quot;</td>
<td>47</td>
<td>-25.6</td>
</tr>
<tr>
<td><em>Myrica pensylvanica</em></td>
<td>C3,&quot;bayberry&quot;</td>
<td>49</td>
<td>-29.5</td>
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<tr>
<td>Marsh Sediment Site 1</td>
<td></td>
<td>4</td>
<td>-16.1</td>
</tr>
<tr>
<td>Marsh Sediment Site 2</td>
<td></td>
<td>18</td>
<td>-15.4</td>
</tr>
<tr>
<td>Marsh Sediment Site 3</td>
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<td>Marsh Sediment Site 4</td>
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<td>24</td>
<td>-17.3</td>
</tr>
<tr>
<td>Waterborne Detritus 1</td>
<td></td>
<td>0.12 µg C/ml</td>
<td>-22.3</td>
</tr>
<tr>
<td>Waterborne Detritus 2</td>
<td></td>
<td>0.22 µg C/ml</td>
<td>-19.1</td>
</tr>
</tbody>
</table>
Figure 5: wax concentrations in five samples
Figure 6: relative compound class abundances for all samples
Figure 7: within class chain length distributions for the n-alkanes
Figure 8: within class chain length distributions for the n-alcohols
Figure 9: within class chain length distributions for the n-acids
Table 2: summary of model results. Tolerance here signifies that the IsoSource program would search for combinations that matched the sediment within 8% relative abundance. The $r^2$ value for the SSD model is for a plot of the modeled vs actual sediment n-alcohol and n-acid distributions.

<table>
<thead>
<tr>
<th>Method</th>
<th>Ammophila</th>
<th>S. alterniflora</th>
<th>S. patens</th>
<th>Baccharis</th>
<th>total C3</th>
<th>total C4</th>
</tr>
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<tbody>
<tr>
<td>bulk carbon</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>32</td>
<td>68</td>
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<tr>
<td>IsoSource range, tolerance = 8</td>
<td>12 to 26</td>
<td>0 to 7</td>
<td>62 to 71</td>
<td>4 to 18</td>
<td></td>
<td></td>
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<td>IsoSource concentration adjusted means</td>
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<td>2</td>
<td>79</td>
<td>13</td>
<td>19</td>
<td>81</td>
</tr>
<tr>
<td>sum squared difference, $r^2=0.85$</td>
<td>0</td>
<td>0</td>
<td>68</td>
<td>32</td>
<td>32</td>
<td>68</td>
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