GEOCHEMISTRY OF LIGNIN BIOMARKERS IN MARSH ENVIRONMENTS OF THE GEORGIA COAST

by

Kathy Mae Loftis

(Under the Direction of Ming-Yi Sun)

ABSTRACT

Lignin biomarkers and their stable-carbon isotopic compositions have been used to detect vascular plant inputs in marine sediments, as well as to quantify the relative contribution of plants using either the C_3 or the C_4 photosynthetic pathway. However, $\delta^{13}C$ values of lignin biomarkers in environmental sediment samples exhibit disparate trends among individual compounds (Goñi et al., 1997; Goñi et al., 1998). It is unclear if either the temporal variability of lignin $\delta^{13}C$ values in source plants, or the effect of degradation on the compound-specific isotopic compositions of lignin derivatives, may result in the observed variability. We set out to address the underlying causes.

The organic matter dynamics in salt marsh environments were investigated by following temporal variations of bulk organic matter parameters, lignin-derived phenols and their compound-specific stable carbon isotopic compositions in salt marsh plants and sediments.

Our next study assessed the impacts of plant material decomposition on geochemical characteristics including molecular isotopic signals. Leaves from two plants,

Spartina alterniflora and *Juncus roemarianus*, were separately incubated under three different environmental conditions: in constantly oxic seawater, in surface (0-5 cm) and subsurface (>10 cm) sediments of a salt marsh in Sapelo Island, Georgia, USA.

We then present a mixing model that incorporates multiple parameters, with the goal more accurately estimating the combination of end-members to a mixture. We show that multi-parameter mixing models require the use of parameters that ensure mass conservation, which complicates the use of elemental ratios and δ values in mixing models. In a reanalysis of literature data on organic matter cycling, we investigated the impact of parameter choices and treatment on the interpretation of the observational data.

Finally we conducted an in situ incubation experiment in a natural salt marsh environment to investigate how the bacterial communities in surface (0 – 5 cm) and subsurface (>10 cm) sediments vary during decay of *S. alterniflora*. Two approaches were used to bin sequences and describe the bacterial communities: (1) the operational taxonomic units (OTUs) based on 97% similarity clustering, and (2) the taxonomic classification using RDP.

INDEX WORDS: Salt marsh higher plants and sediments, Temporal variations, Lignin-derived phenol compounds, Compound-specific isotope ($\delta^{13}C$), Degradation, Mixing models, Microbial community

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DEDICATION

This work is dedicated to those who filled my world with wonder, curiosity, and adventure, to those who taught me to always seek truth and knowledge, to those who instilled in me the love for learning and for life, to my family.

To my parents, Nora and James Loftis, and to my querido Abuelo, los quiero de aquí a la luna, de una estrellita a otra, hasta el final del universo.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. Background

By definition, terrestrial organic matter (TOM) encompasses a multitude of materials originating in the terrestrial ecosystem; however, vascular plants are the dominant primary producers on land, contributing 55 Gt C yr^{-1} (Melillo et al., 1993), and constitute the second largest reservoir of reactive organic carbon (early Holocene estimates) (Hedges, 1992). The flux of TOM being delivered to the ocean by rivers has been estimated at 0.4 – 0.43 Gt C yr^{-1}, and the burial of global organic carbon in modern marine sediments has been estimated at roughly 0.1 – 0.2 Gt C yr^{-1} (Hedges and Keil, 1995). The input of TOM to marine sediments by rivers, alone, exceeds sedimentation by a factor of two, suggesting at least 50% of this recalcitrant pool is remineralized at ocean margins (Hedges et al., 1997). Although the bulk of terrestrially derived material is considered to be recalcitrant, measurements based on biomarkers and stable carbon isotopes indicate a weak TOM signal in marine sediments (Hedges et al., 1997) and in the oceanic dissolved organic pool (Opsahl and Benner, 1997). More recent studies (Goñi et al., 1998; Onstad et al., 2000; Gordon and Goñi, 2004) suggest that offshore transport of

highly degraded, lignin-poor, carbon isotope heavy ($\delta^{13}C \approx -20‰$) soil led to previous underestimations of the terrestrial organic carbon content in marine sediments.

TOM must be identified and differentiated from other organic matter pools as the first step in assessing its fate in marine environments. Stable carbon isotope analysis is a powerful tool for identifying organic matter. Smith and Epstein (1971) demonstrated the ability to categorize land plants into two metabolic groups based on the differential fractionation of ^{13}C due to the use of ribulose bisphosphate (C_3) versus the phosphoenol pyruvate (C_4) photosynthetic pathway. Unidentified plant matter, then, can theoretically be detected, based on its stable carbon isotope composition. However, several caveats arise when applying isotopic analysis to complex environmental samples in an effort to differentiate the various contributing sources of organic carbon.

One of these drawbacks is the inability to differentiate between mixtures of C_3 and C_4 terrestrial plants and marine inputs, as the carbon isotope signal of a mixture containing equal parts of C_3 and C_4 produces a signal similar to that of marine organic matter. For instance, the average $\delta^{13}C$ values for plants utilizing the C_3 pathway are -29‰ to -35‰ (Goñi and Eglinton, 1996) while C_4 marsh plants average -12‰ to -14‰ (Cloern et al., 2002), as a result of the less discriminatory PEP-carboxylase photosynthetic pathway (Fogel and Cifuentes, 1993). Equal contributions of C_3 and C_4 organic matter produce a signal of approximately -21‰. This value falls within the range of macroalgae (-8‰ to -27‰) and marine phytoplankton (-18‰ to -24‰) (Lajtha and Michener, 2007) and obscures the distinction between terrestrial and marine signals.

Further difficulty in identifying components of organic matter results from changes to the bulk organic carbon signal due to the incorporation of material, or the

degradation of material. Macko and Estep (1984) found bacterial cells to be enriched in ^{13}C relative to the carbon substrate, as a result of respiring ^{13}C-depleted CO_2. Incorporation of this enriched carbon signal into the sedimentary record potentially masks the isotopic signal of other organic components. Another difficulty in bulk-sediment isotopic carbon analysis is the preferential degradation of more labile plant constituents. Benner et al. (1987) reported on the implications of using the stable carbon isotopic composition to trace carbon flow. The $\delta^{13}C$ values of polysaccharides and lignin, from the marsh plant *Spartina alterniflora* were monitored throughout a degradation study. Fractionation during the biosynthesis of lignin precursors causes lignin to be depleted in ^{13}C relative to hemicellulose and cellulose. As the polysaccharides were preferentially degraded, the overall carbon signal became more depleted in ^{13}C.

The ability to differentiate the various sources of organic matter is improved through biomarker analysis coupled to compound-specific isotopic analysis. Molecules unique to vascular plants are practical biomarkers for identifying TOM in a mixture of terrestrial and non-terrestrial organic matter. Lignin is a polyphenolic macromolecule with a high chemical stability and is one of the major components of vascular plants, second only to polysaccharides. Lignin is unique to vascular plants and virtually absent in other organisms (Lewis and Yamamoto, 1990); therefore, a significant contribution of vascular plants to total biomass in marine sediments is indicated by a high abundance of lignin. Additionally, the compositional pattern of lignin-derived compounds differs for angiosperm, gymnosperms, woody, and non-woody tissues (Hedges and Mann, 1979a).

Lignin $\delta^{13}C$ analysis is utilized to overcome the complexity of bulk carbon isotope analysis for identifying TOM. Compound-specific isotopic analysis of lignin in

sediments yields a more specific parameter for determining the content of complex organic mixtures. CuO oxidation of plant tissues, sediments, dissolved organic matter, and particulate organic matter yields monomeric and dimeric lignin-phenols that, upon derivitization, can be analyzed by gas chromatography (Hedges and Ertel, 1982). The $\delta^{13}C$ values of lignin derivatives in sediments are used to distinguish C_3 and C_4 vascular plant inputs (Goñi and Eglinton, 1996). This application provides a method for identifying and tracing TOM as it is transported and deposited offshore (Goñi et al., 1997; 1998). A combined approach of analyzing the lignin-phenol chemical composition and stable carbon isotopic signal of the lignin oxidation products provides the ability to distinguish vascular plant organic matter and determine the relative contribution of C_3 and C_4 vascular plants.

Submolecular analysis of lignin generates parameters that further characterize the vascular plant material in a mixture. Chemical oxidation causes the dissociation of the lignin polymer into characteristic phenol monomers that can be grouped into four structural families: p-hydroxyl, vanillyl, syringyl and cinnamyl phenols (Hedges and Mann, 1979a). Total syringyl to total vanillyl phenols (S:V), distinguishes between angiosperms and gymnosperms (Hedges and Mann, 1979a). The usefulness of this parameter is based on the fact that syringyl phenols are produced only by angiosperms, while vanillyl phenols are produced by both angiosperms and gymnosperms. The ratio of total p-coumaric and ferulic acids to vanillyl phenols (C:V), another lignin parameter, can be used to differentiate between woody and non-woody tissues (Hedges and Mann, 1979a). By combining stable carbon isotopes with lignin-phenol composition, new insights into the processes affecting organic matter, such as degradation, can be obtained.

Lignin is subject to changes during degradation (Hedges et al., 1988; Benner et al., 1991; Opsahl and Benner, 1995) and the composition of lignin biomarkers from a degraded sample does not necessarily reflect the original composition of the source plant tissue. Under aerial conditions, increases in $(Ad/Al)_v$ occur during degradation (Hedges et al., 1988). Photochemical degradation of lignin also results in a significant increase in the $(Ad:Al)_v$ ratio (Opsahl and Benner, 1998).

Lignin can be degraded through various pathways. Aromatic ring cleavage results in the general decrease of all lignin phenols. Alternatively, side chains on the lignin units may be cleaved, leaving behind a carbonyl functional group that, upon analytical oxidation, is converted into a carboxyl group, yielding benzoic acids (Crawford, 1981). An increase in benzoic acids, relative to other functional classes, indicates side-chain oxidation (Figure 1.1). The parameters, $[(Ad/Al)_v]$ and $[(Ad/Al)_s]$, are measures of the relative proportion of benzoic acids to aldehydes for vanillin and syringaldehyde, respectively (Hedges et al., 1988). Demethylation can also occur during lignin degradation, affecting those families with methoxylated phenols (vanillyl and syringyl; Figure 1.1). Once demethylated, the compounds are converted into diacids and lost from the analytical window. Lack of methoxyl functional groups on the *p*-hydroxy phenol family, leaves this group untouched. A relative increase in *p*-hydroxyl phenols, then, indicates that demethylation has occurred. This can be calculated using the [P/(S+V)] parameter. Because *p*-hydroxy phenols have non-lignin sources as well, the source of *p*-hydroxy phenols must be determined prior. Of the *p*-hydroxyl phenols, *p*-hydroxyacetophenone is only found in lignin. The ratio of *p*-hydroxyacetophenone to

total *p*-hydroxyl phenols, PON/P, provides source information for the *p*-hydroxyphenols. A decrease in PON/P indicates a non-lignin input.

Changes in isotopic composition, resulting from microbial degradation, have been observed in a variety of chemical compounds and classes. The effect of microbiological transformations on organic matter is complex and the presence and the direction of fractionation are dependent on the molecule and environmental factors (Macko et al., 1994; Meckenstock et al., 1999; Sun et al., 2004; Teece and Fogel, 2007). In a laboratory incubation, bacterial degradation of toluene caused positive isotopic fractionation in the remaining pool (Meckenstock et al., 1999). A laboratory incubation of amino acids resulted in a more complicated picture (Macko and Estep, 1984). In this study, large changes in the isotopic composition of amino acids occurred during early diagenesis, with one group becoming more enriched and another becoming more depleted in ^{13}C, and yet another group remained unchanged. Similar varied fractionations of stable carbon isotopes during degradation were observed for lipids (Sun et al., 2004) and carbohydrates (Teece and Fogel, 2007). The wide-range of trends observed in the $\delta^{13}C$ values of various biomarkers demonstrates the importance of refining our understanding of the effects of degradation on the stable-carbon isotopes of lignin, if we are to apply this technique in discriminating lignin sources and transformations.

Lignin can be altered physically through photochemical degradation (Opsahl and Benner, 1998; Hernes, 2003) and biologically through microbial degradation (Benner, Newell, et al., 1984; Benner et al., 1986; Hedges et al., 1988). Both fungal (Blanchette, 1984; Kirk and Farrell, 1987) and bacterial (Benner, Newell, et al., 1984; Vicuna, 1988; Alexandre and Zhulin, 2000) degradation of lignin occurs in aerobic environments.

Although the identified lignin-degrading enzymes require the presence of oxygen (Kirk and Farrell, 1987; Popp et al., 1990), studies suggest that the degradation of lignin can also occur biotically under anoxic conditions (Benner, Maccubbin, et al., 1984; Colberg and Young, 1985).

Using radio-labeled synthetic lignin, Benner et al. (Benner, Maccubbin, et al., 1984) monitored the evolution of $^{14}CO_2$ during the anoxic degradation of lignin and determined that approximately 2% hardwood- and 17% herbaceous-derived lignin were anaerobically remineralized in one year. An incubation study utilizing lignin-derived phenols to monitor the degradation of belowground biomass lignin in anoxic sediments confirmed previous mineralization rates (Benner et al., 1991). Through biomarker analysis Dittmar and Lara (2001) determined that in sulfate reducing sediments, lignin concentrations decreased more rapidly relative to total organic matter. Despite current knowledge of the potential for lignin degradation in anoxic sediments, very little is known about the microorganisms capable of degrading lignin under these conditions.

The complex structure of lignin sets constrains on the enzymes capable of degrading it (Jeffries, 1994). Organisms containing the enzymes capable of degrading lignin can convert the macromolecule to acid-precipitable polymeric lignin (Crawford, 1981), and ultimately to CO_2 (Gonzalez et al., 1997). Lignin degradation rates are stimulated by molecular oxygen (Kirk and Farrell, 1987). Therefore, lignin degradation under anaerobic conditions is not anticipated. However, rumen bacteria are found to be capable of anaerobic lignin degradation. Chen and others (1985) observed cleavage of β-arylether bonds, the dominant interpolymeric linkage in lignin, by rumen bacteria under anoxic conditions. More recently, cleavage of benzyl ether bonds in lignin have also been

documented (Kajikawa et al., 2000). These bonds serve as cross-linkages between lignin and polysaccharide complexes, resulting in steric hinderance to enzymatic attack on these polymers (Jeffries, 1994). Ko et al. (2009) monitored lignin degradation in sulfate reducing environments and detected the formation of polymeric soluble lignin. Subsequently, aromatic compound concentrations increased with a concurrent decrease in polymeric soluble lignins, suggesting degradation of this fraction into aromatic compounds. Further anaerobic degradation of the resulting aromatic substrates, which does not require molecular oxygen (Fuchs, 2007), has been linked to methanogenic consortia (Healy et al., 1980). These studies suggest that acetate, formed during intermediate steps and as an end product of anaerobic aromatic degradation, is made available to methanogens. Anaerobic lignin degradation in coastal salt marshes may be initiated by the depolymerization of lignin and formation of low molecular weight soluble polymers. Bacterial degradation of lignocellulose predominates that of fungal degradation in salt marsh sediments (Benner, Newell, et al., 1984), therefore these low molecular weight, soluble polymers may then be degraded, through a series of steps, to volatile fatty acids by a consortium of bacteria.

Bacterial and fungal degradation causes diagenetic alterations to the lignin molecule, thereby affecting the composition of lignin derivatives. These alterations have been used to quantify the diagenetic state (i.e. fresh or degraded material) of lignin in the environment (Gordon and Goñi, 2004). Whether these diagenetic alterations also affect the stable carbon isotopic composition of lignin and lignin derivatives is currently unknown.

2. Hypotheses and Objectives

The main hypotheses for this work are: 1) the isotopic compositions ($\delta^{13}C$) of lignin biomarkers in plants vary temporally due to changes in environmental conditions and metabolic pathways; 2) degradation of vascular plant organic matter alter the stable carbon isotopic compositions of lignin-derived phenol compounds in sediments due to involvement of different degrading pathways; 3) lignin degradation, and consequent alteration of chemical and isotopic signals, in salt marsh sediments depend on environmental redox conditions and related microbial communities. To test these hypotheses, a series of field measurements, in situ incubations and laboratory experiments were conducted. The specific objectives were:

1) Assess temporal variations in lignin compositions and in $\delta^{13}C$ signatures of lignin derived compounds in marsh plants (*S. alterniflora and J. roemarianus*) and ambient sediments, and apply that knowledge to gain a better understanding of the carbon dynamics in marsh sediments;
2) Examine lignin degradation and its impact on the molecular isotopic signals of lignin-derived phenols by conducting an in situ litterbag incubation of two marsh plants (*S. alterniflora* and *J. roemarianus*) in Georgia coastal marsh sediments under oxic and anoxic conditions;
3) Develop a mixing model that was capable of integrating various chemical characteristics to accurately deduce the proportion of end-members contributing to a mixture
4) Describe the bacterial communities associated with plant litter and lignin degradation in anoxic and oxic sediments by conducting 16S rRNA gene

sequencing and $\delta^{13}C$ analysis of bacterial phospholipid fatty acid (PLFA) for litterbag samples.

3. Organization of this Dissertation

In Chapter 2, I investigate the organic matter dynamics in a salt marsh by assessing the temporal variation in bulk tissue parameters, lignin phenol compositions, and the carbon isotope composition of lignin-derived monomers, for salt marsh plants, *Spartina alterniflora* and *Juncus roemarianus* and sediments from two sites in a coastal Georgia, USA salt marsh. The two sites were chosen based on differences in the flooding regime, as well as differences in dominant macrophyte. Sediments were collected from a creek-bank site, where *S. alterniflora* is dominant, and a high-marsh site, where *J. roemarianus* is dominant.

In Chapter 3, I quantify the rate of degradation, in concert with the alteration of geochemical characteristics of decomposing plant material. *Spartina alterniflora* and *Juncus roemarianus* leaves were incubated under three different environmental conditions over one year to examine the effect of decomposition on the lignin and isotopic compositions.

In Chapter 4, I show that a multi-parameter mixing model used to elucidate the relative contribution of organic matter materials to a mixture requires the use of parameters that ensure mass conversation, thereby barring the use of δ values and elemental ratios

In Chapter 5, I explore the distribution of bacterial communities associated with *S. alterniflora* leaf litter deposited in surface sediments (0-5 cm) and in sub-surface (>10 cm) sediments, during different stages of decomposition.

Chapter 6 summarizes the findings from the main chapters and links together the major concepts from dissertation to provide a clearer understanding of organic matter cycling in coastal systems.

Figure 1.1. Examples of how a guaiacyl-based lignin unit would be altered as a result of the principal lignin degradation pathways. Aromatic ring cleavage, side-chain oxidation, and demethylation pathways are depicted and the resulting changes to the lignin degradation parameters is denoted. Modified from (Filley et al., 2000)

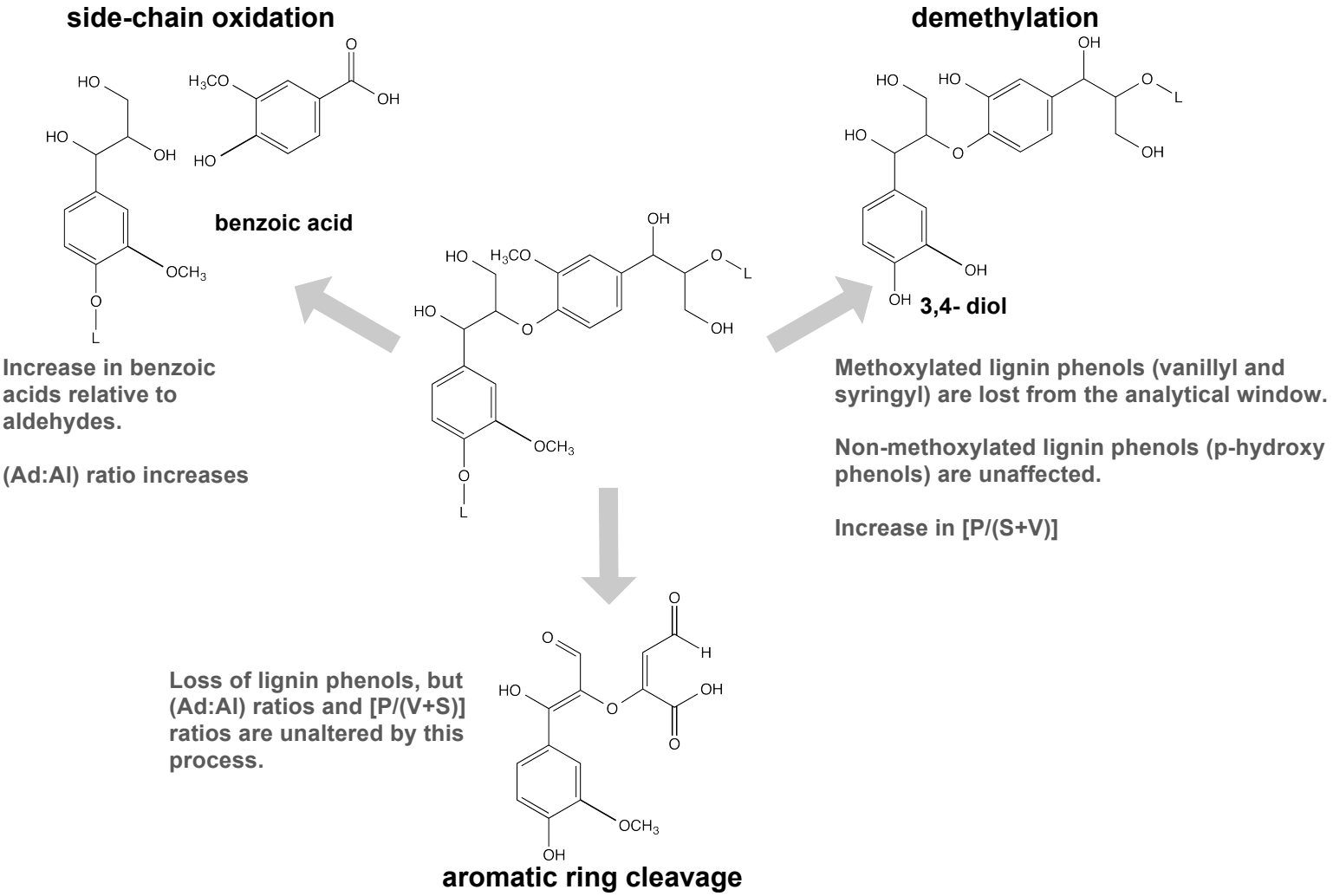
side-chain oxidation
benzoic acid
Increase in benzoic acids relative to aldehydes.
(Ad:Al) ratio increases
demethylation
3,4- diol
Methoxylated lignin phenols (vanillyl and syringyl) are lost from the analytical window.
Non-methoxylated lignin phenols (p-hydroxy phenols) are unaffected.
Increase in [P/(S+V)]
Loss of lignin phenols, but (Ad:Al) ratios and [P/(V+S)] ratios are unaltered by this process.
aromatic ring cleavage

Figure 1.1

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CHAPTER 2

TEMPORAL VARIATIONS OF BULK ORGANIC MATTER PARAMETERS, LIGNIN-DERIVED PHENOLS, AND THEIR COMPOUND-SPECIFIC STABLE CARBON COMPOSITIONS IN GEORGIA SALT MARSH ENVIRONMENTS[1]

[1] Loftis, K., M-Y. Sun. To be submitted to Geochimica et Cosmochimica Acta

Abstract

This study was designed to investigate organic matter dynamics in salt marsh environments by following temporal variations of bulk organic matter parameters (OC%, TN%, $\delta^{13}C_{TOC}$, $\delta^{15}N_{TN}$, and C/N ratio), lignin-derived phenols and their compound-specific stable carbon isotopic compositions in salt marsh plants and sediments. *Spartina alterniflora* (C_4 plant) and *Juncus roemarianus* (C_3 plant) leaves, and ambient surface sediments were collected monthly over one year from two neighboring sites: a creek-bank and a high-marsh, in Georgia, USA. Plant samples were analyzed for temporal variations between spring/summer and fall/winter, and sediment samples were analyzed for differences between periods of low and high river discharge in the neighboring Altamaha River, as well as spatial differences between the two sites.

TOC content of *S. alterniflora* did not vary temporally; however, TN content was greater in the spring/summer than in the fall/winter, when the plants were in a state of senescence. Two lignin-derived phenols, vanillic acid and syringic acid, exhibited statistically significantly different $\delta^{13}C$ values between the two periods, with higher values observed during fall/winter. *J. roemarianus* exhibited distinctly different temporal trends than *S. alterniflora.* The TOC content of *J. roemarianus* was significantly greater during the spring/summer, but the TN content did not exhibit significant differences between the two periods. Likewise, the $\delta^{13}C$ values of lignin-derived phenols did not exhibit clear temporal trends. While clear temporal trends were not evident, for most lignin phenols in *S. alterniflora*, or for any lignin phenols in *J. roemarianus*, the $\delta^{13}C$ values did vary considerably during the study period. Vanillin exhibited the least

variation in carbon isotope composition in both *S. alterniflora* (7‰) and in *J. roemarianus* (4‰).

At the creek-bank site, during periods of high discharge, chemical characteristics and total organic carbon isotope composition indicated deposition of fresh, non-woody plant material during this period. As the season progressed, and the period of low discharge approached, degradation of the plant material that was deposited during the high discharge period was evident. At the high marsh site, during periods of high discharge, chemical parameters indicated the deposition of carbon-rich, non-woody angiosperm tissue.

We observed that organic matter dynamics at two different marsh sites were affected by different processes. Sediments in the creek-bank were dominated by inputs of vascular plant matter from both C_3 and C_4 organic matter sources, which are deposited during periods of high-discharge. They are subsequently degraded, altering the geochemical signals in the sediment. The high-marsh site sediments were composed predominately of C_3 vascular plant inputs, most likely from *J. roemarianus*.

1. Introduction

The transfer of organic matter (OM) from land to ocean is an important process in the global biogeochemical cycle of carbon, providing a conduit for oceanic burial and preservation of terrestrial primary production. At the transition of the land and ocean interface, salt marshes represent highly productive ecosystems with major contributions from autochthonous production of vascular plants (Gallagher et al., 1980; Darby and Turner, 2008), and additional contribution from phytoplankton (Gallagher, 1975; Haines,

1977), benthic algae, bacteria, and allochthonous inputs of vascular plants. *Spartina alterniflora* and *Junus roemarianus* are the dominant macrophytes in Georgia salt marshes, annually producing up to 3700 g/m^2 and 2200 g/m^2, respectively, in above-ground biomass. Belowground biomass of *S. alterniflora* exceeds that of aboveground, annually producing 7,620 g/m^2 (Schubauer and Hopkinson, 1984). Ultimately, this biomass is converted to particulate organic matter, detritus. The importance of detritus in Georgia salt marshes has long been understood (Teal, 1962; Odum and la Cruz, 1963), as the fate and transformation of this detritus has important implications for the salt marsh as well as adjacent coastal areas (Moran et al., 1991; Dame and Allen, 1996). Accurately estimating of the magnitude of fluxes and sinks, as well as understanding of the processes that control carbon transport and transformation, are key components for resolving issues of coastal carbon cycling and ultimately constraining the global carbon cycle.

There are various biochemicals unique to vascular plants that can be used as molecular tracers; of these, lignin is commonly used to detect vascular plant organic matter. Lignin is an abundant component of vascular plant tissue, second to cellulose (Boerjan et al., 2011), accounting for as much as 20% to 30% of vascular plant tissue. This aromatic biopolymer forms the secondary xylem in woody trees and the reinforcement in non-woody, herbaceous plants. Currently, there is no method that can isolate lignin while maintaining its native structure. Instead, lignin must be broken down into smaller units. One method copper oxide (Hedges and Ertel, 1982). This method produces, amongst many oxidation products, eight monomers that can be grouped as vanillyl (V), syringyl (S), and cinnamyl (C) phenols. The relative distribution of these monomers can impart information on the source of vascular plant organic matter and can

also be used to distinguish a breadth of tissue types (Hedges and Mann, 1979a). For example, the lignin phenol compositions provide information on the relative abundance of gymnosperm versus angiosperm and woody versus non-woody tissue (Hedges and Mann, 1979a).

Additionally, the carbon isotope composition of lignin phenols can be measured by compound-specific isotopic analysis using an isotope ratio mass spectrometer (Goñi and Eglinton, 1996). Analysis of the carbon isotope composition of lignin phenols provides information on the plant type because differences in photosynthetic pathways between plants utilizing the Calvin Cycle (C_3) and the Hatch-Slack metabolism (C_4) are recorded in the carbon isotope composition of plant biomass and biomarkers, including lignin. Carbon isotope analysis of lignin phenols, then, provides an advantage to the carbon isotope analysis of bulk organic matter in environmental samples in that the organic molecules from specific vascular plant source can be exclusively tracked. Inference on plant type distribution from the carbon isotope composition of lignin phenols requires that the isotopic signature of the source plant be preserved despite variations resulting from environmental effects, metabolic synthesis, and degradation. Tracing the source of the lignin phenols then requires not only that the lignin phenol $\delta^{13}C$ values remain unchanged, but also that the carbon isotope composition of the source plants remain fixed. However, at present, few studies have shed light on whether these molecular and isotopic signals vary from production stage of source plants to ultimate preservation in sediments.

Compound-specific lignin-phenol analysis of several plant species showed intermolecular variability, which may be explained by biosynthetic pathway (Goñi and

Eglinton, 1996). For instance, cinnamyl acids were found to be more enriched in ^{13}C than other lignin-phenols (Goñi and Eglinton, 1996). As cinnamyl phenols are intermediaries in the production of lignin biosynthesis, the authors attributed differences in carbon isotope composition to a less tortuous pathway leading to the formation of cinnamyl phenols. Intermolecular variability may have implications in deducing source contributions to natural mixtures. When the $\delta^{13}C$ signal of sediments and plants were compared to determine lignin sources in the sediments (Goñi and Eglinton, 1996), the isotope composition of most lignin-phenols in the sediments were nearly identical with the exception of vanillic acid, which was more depleted in ^{13}C During decomposition of lignin, vanillic acid is produced from side-chain oxidation of vanillyl phenols (Hedges et al., 1988). The presence of degraded C_3 material could contribute sufficient vanillic acid that it would dominate the vanillic acid pool (Goñi and Eglinton, 1996), and thus affect the carbon isotope composition of the one phenol discriminately. However, temporal variability within a single plant species, affecting intermolecular isotope $\delta^{13}C$ values differently, cannot be ruled out. When the $\delta^{13}C$ values of various lignin phenols were measured along a transect in the Gulf of Mexico, one lignin phenol (vanillin) was depleted relative to the other lignin compounds (Goñi et al., 1997). This could be caused by at least two processes: the input of gymnosperm wood, which would only contribute lignin phenols to the vanillyl pool, imparted a signal to the vanillin pool (Goñi et al., 1997); or intramoecular heterogeneity in ^{13}C composition of lignin phenols in the source plants resulted in variable $\delta^{13}C$ values between lignin phenols with the same origin.

The goals of this study are: 1) to assess the temporal variability of lignin phenol composition and the carbon isotope composition of lignin in plants with differing

metabolic pathways, *S. alterniflora* (C_4) and *J. roemarianus* (C_3); 2) to characterize sedimentary lignin compositions and determine their molecular stable carbon isotopic signals in two depositional sites; and 3) to identify the sources of organic carbon in these two different salt marsh sediments.

2. Materials and Methods

2.1. Study site

Samples were collected from two sites in salt marsh environments on the coast of Georgia, USA (Figure 2.1). Two sites with distinct macrophyte distributions were selected for sampling. One site was located along a creek-bank that was vegetated and had dense growth of tall form *S. alterniflora*. A second site was located in the higher marsh. This site had dense stands of *J. roemarianus.* The marshes were adjacent to the Doboy sound, an estuary that receives considerable input of freshwater from local runoff and input of the Altamaha River. Although the sound is marine-dominated, during high discharge periods, the salinity at Hudson creek dropped to 17.9 (Figure 2.2) indicating freshwater influence. The salinity at the creek site was highly correlated to periods of high discharge for the Altamaha River ($r = 0.76$; $p < 0.01$). Altamaha River discharge and Hudson Creek salinity data were provided by Daniela DiIorio in conjunction with the Georgia Coastal Ecosystems LTER and the United States Geological Survey (GCE-LTER; USGS).

2.2. Sampling description and sample classification

Sediment and plant leave samples were collected on a monthly basis between November 2005 and May 2007. Sediment from the top 0.5 cm, at either the creek-bank

site or the high-marsh site, was collected and kept frozen until analysis. *S. alterniflora* leaves were collected from the creek-bank site, while *J. roemarianus* leaves were collected from the high-marsh site. Senescent or fresh leaves, depending on the season, were collected and kept frozen until analysis. *S. alterniflora* leaves were always collected from the upper portion of the shoots and thus represented the newest growth, however if only senescent leaves were present, these were collected.

Two distinct sampling periods were defined for both plants and sediments. Sediment sampling dates were defined as either collected during high discharge period (December 2005 – April 2006 and January 2006 – April 2007) or low discharge period (November 2005 and May 2006 – December 2006), based on the Altamaha River discharge flow rates (Figure 2.2). Plant collection dates were separated into spring/summer (April-September) and fall/winter (October-March) to follow periods of growth and senescence for *S. alterniflora* and *J. roemarianus* (Giurgevich and Dunn, 1982; Schubauer and Hopkinson, 1984; Darby and Turner, 2008).

2.3. Elemental analyses and bulk sample isotope measurements

Freeze-dried and ground plant tissues were analyzed without acidification for total organic carbon, total nitrogen content, total organic carbon stable isotope composition, and total nitrogen stable isotope composition by in-line combustion on an elemental analyzer (Carbo Erba CHN analyzer) coupled to an isotope mass spectrometer. Sediment samples were freeze-dried, ground and acidified with HCl prior to elemental analysis. Total organic carbon (TOC), total nitrogen (TN), $\delta^{13}C$ of total organic carbon, and $\delta^{15}N$ of total nitrogen were measured using the same analyzer coupled to an isotope mass spectrometer.

2.4. Lignin-phenol analysis

Freeze-dried plant and sediment samples were analyzed for lignin-derived phenols using a modified cupric oxide method (Goñi and Montgomery 2000; Hedges and Ertel 1982). Briefly, freeze-dried plants or sediments were oxidized with CuO in alkaline conditions (2 N NaOH) with $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in stainless steel reactor vessels at a temperature of 150 °C for 3 hours. The products from the reaction were extracted with ethyl acetate. Subsequently, the extracts were reacted with BSTFA + 1% TCMS by heating at 60 °C for 10 min to convert phenols (with polar OH groups) to trimethylsilyl derivatives (more thermally stable) that can be analyzed readily via gas chromatography. Trimethylsilyl derivatives were analyzed by gas chromatography using a 30 m x 0.250 mm (i.d.) column (0.25 μm film thickness) column fitted to a flame ionization detector. Quantification of lignin-derived phenols was conducted based on comparison with the recovery standards ethyl vanillin and *trans*-cinnamic acid and their response factors relative to each individual compound.

The total lignin content of samples normalized to 100 mg OC (Λ_8), and the total syringyl and vanillyl lignin content (Λ_6, normalized to 100 mg OC) were calculated. In addition, several lignin parameters, which can provide additional information on the nature of the vascular plant organic matter, were estimated. The ratio of syringyl (syringaldehyde, syringic acid, and acteosyringone) to vanillyl (vanillin, vanillic acid, and acetovanillone) phenols (S/V) and the ratio of cinnamyl (p-hydroxycinnamic acid and ferulic acid) to vanillyl phenols (C/V) were used to determine the relative contribution of angiosperm versus gymnosperm and woody versus non-woody tissue, respectively (Hedges and Mann, 1979b). However, these ratios are influenced by fungal degradation

(Hedges et al., 1988), subaqueous degradation (Haddad et al., 1992; Opsahl and Benner, 1995), sedimentary degradation (Benner et al., 1990), and photochemical degradation (Opsahl and Benner, 1998; Hernes, 2003). In addition, the ratio of vanillic acid to vanillin [$(Ad/Al)_V$] and syringic acid to syringaldehyde [$(Ad/Al)_S$] are also diagenetic indicators (e.g. (Opsahl and Benner, 1995). During oxidative degradation, the side chain of vanillyl and syringyl units can be oxidized yielding carbonyl carbons that are easily further oxidized to carboxylic acids. As a result, during oxidative degradation, the (Ad/Al) ratios of vanillyl and syringyl moieties gradually increased.

2.5. Lignin-derived phenol compound-specific isotope analysis

The stable carbon isotope composition of individual lignin phenols was determined by gas chromatographic isotope ratio mass spectrometry (GC-IRMS). Analysis was performed on a GC combustion system coupled to a Delta II isotope ratio mass spectrometer. Accuracy and reproducibility of the instrument was monitored through internal standard monitoring using 3,4-dihydroxybenzoic acid as an internal standard. The stable isotope composition of the BSTFA-TMS carbon was measured off-line and used to correct the $\delta^{13}C$ value of lignin phenols (Goñi and Eglinton, 1996).

2.6. Principal components analysis (PCA)

The complexity of the sediment datasets was reduced by performing PCA analysis using the princomp function in MATLAB (MathWorks, Inc., 2009). Principal component analysis reduces multivariate systems into fewer dimensions by finding the redundancy in measured variables and grouping them into a new variable. This method generates a set of variables, principal components (PC) that are a linear combination of the original. Plots can be limited to the first few PC if a significant amount of the

variance in the original dataset is encapsulated. The loadings, representing the correlation between the variables measured in the sediment and the principal component scores were calculated. The PC scores of each sediment sample were then calculated, indicating the relative influence of each PC on the sample. PCA analysis was performed using the pca function in MATLAB (MathWorks, Inc., 2009).

3. Results

3.1. Temporal and spatial variations in bulk elemental parameters and $\delta^{13}C$

The TOC contents of *S. alterniflora* leaves did not vary considerably during the study period, ranging between 37% and 43%, and did not exhibit significant changes between the two, spring/summer or fall/winter, seasons (Table 2.1). On the other hand, TN contents differed significantly ($p< 0.5$; Table 2.1), likewise affecting C:N ratios, between seasons, with higher TN observed in spring/summer than in fall/winter. TN in *S. alterniflora* exhibited a strong, positive correlation to temperature ($R = 0.6$, $p < 0.05$). The TN content in *J. roemarianus* did not differ significantly between seasons however, the TOC content was greater in the spring/summer than in the fall/winter ($p < 0.05$; Table 2.1), also affecting C:N ratios.

The TN and C:N ratios in creek-bank sediments varied differently with discharge periods (Table 2.1), with greater TN content and lower C:N ratios in high discharge periods ($p < 0.05$). High-marsh sediments did not experience statistical differences in their TN or C:N ratios between discharge periods. However, in the high-marsh sediments, TOC was positively correlated with discharge and increased during high discharge. When comparing sediment samples between the two sites from the same time period (May 2006

– May 2007), C:N ratios at the creek-bank site differed from those at the high-marsh site ($p = 0.4$). At the high-marsh site, C:N ratios were greater than those at the creek bank, and TOC and TN were significantly ($p << 0.01$) lower than those at the creek-bank site.

The TOC $\delta^{13}C$ values for either *S. alterniflora* or *J. roemarianus* did not differ statistically between seasons. In the sediments, values were only statistically different between high and low discharge periods at the creek-bank site. At the creek-bank sites, $\delta^{13}C$ values were strongly correlated with discharge, and were more positive during high discharge periods with a difference of 0.8‰ (Table 2.1).

3.2. Temporal and spatial variations in lignin content and parameters

Significant variations between spring/summer and winter/fall were not observed for individual lignin phenol concentrations, or in the total lignin content (Λ_6), for *S. alterniflora* or *J. roemarianus* (Table 2.2), but the lignin content of the two species did differ from one another. When *S. alterniflora* and *J. roemarianus* leaves and spikelet collected from the same time period (May 2006 – May 2007) were compared, the Λ_6 content of *J. roemarianus* was overall greater ($p < 0.05$). When the lignin content of sediments from the creek-bank and high marsh site were compared over the same time range (May 2006-May2007), the sedimentary lignin content was not statistically different between the two sites. Likewise, within each site the lignin content did not vary during high or low river discharge periods.

Lignin parameter ratios were not different temporally within the plants, but there were differences between the plants. In *J. roemarianus*, the S/V ratio was greater than that of *S. alterniflora* (Table 2.3; $p < 0.01$). Although the vanillyl content of both plants was similar, the syringyl content of *J. roemarianus* was twice that of *S. alterniflora*.

Spatial differences in lignin parameters were not evident when comparing the creek-bank and high-marsh sites; however temporal variations within site were apparent.

The lignin parameters exhibited clear temporal trends in sediments at the two sites. At the creek-bank site, C/V ratios were greater during high discharge (Table 2.3; $p < 0.05$). At the high-marsh site, both S/V ratios and C/V ratios differed between temporal periods. During the high discharge period, the S/V ratio and C/V ratios were greater than those in the low discharge period (Table 2.3; $p < 0.05$).

3.3. Temporal and spatial variability in lignin phenol compound-specific $\delta^{13}C$ values

The carbon isotope composition of lignin phenols in *S. alterniflora* did not generally exhibit statistic differences between the spring/summer and fall/winter seasons, with the exception of vanillic acid and syringic acid, which were more enriched in the spring/summer ($p < 0.05$). Though grouping into season obscured the trend, lignin phenols, excluding vanillin and acetovanillone, were also strongly negatively correlated (all $R < -0.6$, $p < 0.05$) with temperature. Lignin phenols in *J. roemarianus,* however, did not exhibit clear trends as there was no correlation with temperature, and no statistical difference between seasons.

Although lacking clear seasonal variation in $\delta^{13}C$ values, there were intermolecular variations in the $\delta^{13}C$ values of lignin phenols in both *S. alterniflora* and *J. roemarianus* (Figure 2.3). The extent of variation differed between plants and between lignin phenols. There was generally more spread in the $\delta^{13}C$ values of lignin phenols in *S. alterniflora*, than the same compound in *J. roemarianus*. The $\delta^{13}C$ of vanillin and p-coumaric acid exhibited the least variation in both *S. alterniflora* and *J. roemarianus.* For both plant species, the lignin phenol $\delta^{13}C$ values were more negative than the TOC $\delta^{13}C$

values, with the exception of the cinnamyl phenols (Figure 2.3). In *S. alterniflora* and *J. roemarianus*, the deviation of the $\delta^{13}C$ signal of lignin phenols from TOC $\delta^{13}C$ follows a similar pattern. The $\delta^{13}C$ of vanillin and ferulic acid are most similar to TOC $\delta^{13}C$, and syringic acid is the most dissimilar. In fact, syringic acid from *S. alterniflora* has the same $\delta^{13}C$ value as *J. roemarianus* TOC $\delta^{13}C$.

The $\delta^{13}C$ of lignin phenols in sediments did not exhibit clear temporal variations, but were spatially distinct between two sites ($p < 0.01$) when the same time period was compared (May 2006 – May 2007). The $\delta^{13}C$ values of all lignin phenols, with the exception of acetosyringone and ferulic acid were distinct and were more positive at the creek-bank site (Figure 2.4) than those at the high-marsh site. Differences in the relationship between $\delta^{13}C$ values of individual lignin phenols in sediments and local macrophytes were also evident. At the creek bank site, the lignin phenol $\delta^{13}C$ values in sediments were generally more negative than those in *S. alterniflora*, the dominant plant species at that site. At the high-marsh sediment, $\delta^{13}C$ values of lignin phenols were generally more positive than *J. roemarianus*, the dominant macrophyte at that site.

4. Discussion

4.1. Variations in lignin-phenol $\delta^{13}C$ values and implications

The carbon isotope composition of lignin phenols provides insight into the type of vascular plant material in environmental mixtures (Goñi and Eglinton, 1996), because plant biomass $\delta^{13}C$ values are dependent on the photosynthetic pathway being expressed by the plant. The carbon isotope signal imprinted during photosynthesis is preserved on the molecular level and in the bulk biomass, with the $\delta^{13}C$ of biomass simply being a

weighted average of all biomolecular components. Separation of C_3 and C_4 plants, based on carbon isotope composition, then, is feasible through analysis of the carbon isotope composition of the total organic carbon, $\delta^{13}C_{OC}$. Based on this principle, plants expressing either a C_3 or a C_4 photosynthetic pathway can be identified as having $\delta^{13}C_{OC}$ values of approximately -30‰ and -15‰ (Smith and Epstein, 1971). Assuming a similar photosynthetic signal in the lignin carbon isotope composition, the relative proportion of different plant types in a mixed medium can be interpreted (Goñi and Eglinton, 1996; Bianchi et al., 2007). Lignin, however, is depleted in ^{13}C relative to TOC (Figure 2.3; Benner et al., 1987), complicating this interpretation. Therefore, a priori knowledge of the $\delta^{13}C$ value of lignin phenols from various plants is necessary to identify the contribution of different plant materials to a mixture.

Throughout the study period, the $\delta^{13}C$ value of individual lignin phenols in *S. alterniflora* and *J. roemarianus* varied to different extents from the TOC $\delta^{13}C$. While the $\delta^{13}C$ values of vanillic acid and syringic acid in *S. alterniflora* overlapped with that of *J. roemarianus* TOC, the carbon isotope signals of vanillin and ferulic acid were similar to the TOC $\delta^{13}C$ signal of each respective plant. The lower carbon isotope variabilities of vanillin and ferulic acid reveal these compounds to be more ideal biomarkers for identifying source materials in a mixture. Cinnamyl phenols (ferulic acid and p-coumaric acid), however, are absent in woody tissues, but vanillyl phenols are ubiquitously found in all vascular plant types and tissues. Despite variability in lignin-phenol $\delta^{13}C$ values within plant tissues, vanillin lends itself as a powerful biomarker for carbon isotope analysis, given the low temporal variability in the $\delta^{13}C$ values, relatively high abundance, and its universal presence in all plant types and structures.

The observed intermolecular differences in the $\delta^{13}C$ values of lignin may help explain previously observed variations in lignin phenol carbon isotope composition (e.g. Goñi and Eglinton, 1996). Differences in the $\delta^{13}C$ value of lignin phenols within a mixture may be the result of differential contribution of individual lignin phenols from different sources with distinct $\delta^{13}C$ signals. On the other hand, we present a scenario where differences could simply result from intermolecular variability in the isotope signal of lignin phenols originating from a single source.

The carbon isotope compositions of the primary compounds produced during photosynthesis are dependent on the photosynthetic pathway employed by the plant species. The Calvin Cycle (C_3), Hatch-Slack (C_4), and the Crassulacean Acid Metabolism (CAM) each result in different fractionation of ^{13}C. These fractionations are based on the diffusion, dissolution and carboxylation steps employed in photosynthesis. O'Leary (1981; 1988) reviewed these processes in detail. The primary compounds produced during photosynthesis are then shuttled to downstream processes (Hayes, 2001) to produce secondary products (e.g. lipids, proteins, and lignin). The processes leading to the formation of secondary products can involve oxidation, reduction, bond formation, and cleavage; each of these scenarios fractionates carbon isotopes as a result of kinetic isotope effects. In addition, the $\delta^{13}C$ of secondary products is also dependent on 1) the carbon flow from reactants to products and 2) on the isotope effect of the reactions (Hayes, 2001). As a result, the $\delta^{13}C$ of products will vary depending on the proportion of reactant converted. Finally, the carbon isotope composition of secondary products may be further affected by their consumption when these compounds act as intermediates in other

reactions. As a secondary product, lignin possesses a carbon isotope composition reflecting a myriad of processes.

Identifying the processes leading to the observed variability in $\delta^{13}C$ values within a plant species is beyond the scope of this paper. However, the strong negative correlation between temperature and the $\delta^{13}C$ value of lignin phenols in *S. alterniflora* suggests that seasonal processes, whether internal (e.g. physiological differences) or external (e.g. degradation), are linked to the observed variations. One explanation for the temporal variation in $\delta^{13}C$ values of lignin phenols is degradation-induced fractionation of carbon isotopes. The $\delta^{13}C$ values of vanillic acid and syringic acid in *S. alterniflora* were more positive during fall/winter, when TN content was lower. Nitrogen content in *S. alterniflora* decreases during periods of senescence, as N is translocated to the rhizome material (Hopkinson and Schubauer, 1984). The increased ^{13}C content of lignin phenols in senescent material suggests degradation effects may have been attributed to the variability. Increases in ^{13}C content of compounds during degradation have been observed (e.g. Meckenstock et al., 1999; Sun et al., 2004; Teece and Fogel, 2007; Liu et al., 2010). One possible explanation for the increased $\delta^{13}C$ values of vanillic acid and syringic acid during the fall/winter may be the preferential degradation of isotopically lighter material. Degradation of *S. alterniflora* in seawater resulted in large variations in the $\delta^{13}C$ value of lignin phenols (see Chapter 2). While the epiphytic organisms and degradation pathways of *S. alterniflora* would differ from those of seawater, degradation induced variations in carbon isotope compositions cannot be ruled out.

4.2. Organic carbon dynamics at two sites in a salt marsh

Different processes controlling organic carbon cycling seem to be occurring at the creek-bank versus high-marsh site. The elemental content, lignin parameters, and $\delta^{13}C$ values of TOC and lignin phenols at the creek-bank sediments are largely controlled by introduction of allochthonous material. On the other hand, these signals at the high-marsh sediments seem to be largely influenced by import and degradation of local produced organic materials.

4.2.1. Creek Bank Site

Together, the values measured for C/N ratios, C/V ratios, and $\delta^{13}C$ of TOC and lignin phenols indicate that the sediments at creek bank site receives a large vascular plant input that is controlled by temporal variability in deposition during different river discharge regimes. At high discharge, C/V ratios increased, C/N ratios were lower, and TOC $\delta^{13}C$ values were more positive than at low discharge.

The higher C/V ratios in creek-bank sediments during periods of high discharge indicated input of fresher (Opsahl and Benner, 1995) and more non-woody material (Hedges and Mann, 1979a). C/V ratios are useful parameters for identifying the source of vascular plant material in mixtures due to its disparate distribution among vascular plant tissues. Cinnamyl phenols (C) are present only in non-woody tissue, whereas they are absent in woody tissues. As such, relatively higher C/V ratios during the high discharge period in creek bank sediments indicated the presence of an increased presence of non-woody tissue. Interpretation of C/V ratios in mixtures, such as sediments is complicated due to an introduction of degraded vascular plant material. During degradation the cinnamyl phenols are preferentially lost, as they are less stable than vanillyl compounds,

and the C/V ratio decreases (Hedges et al., 1988; Haddad et al., 1992; Opsahl and Benner, 1995). Decreased C/V ratios preclude its use for quantitatively determining the proportion of herbaceous tissue, the ratio can be used qualitatively. For example, during low discharge, C/V ratios are lower, but it is unclear if this is due to degradation (Opsahl and Benner, 1997) or incorporation of woody material (Hedges and Mann, 1979a). However, the only process that increases C/V ratio is an increase in non-woody tissue. Therefore, the greater value of C/V observed during the high discharge period suggests fresher and/or more herbaceous plant material is deposited during this period.

The relatively lower C/N ratios during periods of high discharge indicates the introduction of less degraded material (Lehmann et al., 2002). As organic matter is degraded, the more labile, N-rich compounds are preferentially removed, causing an increase in C/N. While a greater contribution of protein-rich algae could also impart a low value in the C/N ratio, dilution of plant matter with a large algal pool would also affect the lignin content of the sediments. Incorporation of a large amount algal biomass would dilute the lignin content and the lignin content of the sediment would decrease since microalgae do not possess lignin. The lack of an observed dilution effect in the lignin content of creek-bank sediments during high-discharge (Table 2.2) suggests algal input is not responsible for the observed signals in C/N ratios or TOC $\delta^{13}C$.

However, it should be noted that the non-vascular component of Creek Bank sediments may be substantial. The lignin content does not fluctuate between high and low-discharge, suggesting that the relative contribution of non-vascular to vascular plants does not seem to be altered between low and high-discharge. However, the lignin content of the sediments is lower than that of plants. Because lignin is refractory, it is expected

that lignin would accumulate in sediments. The low amount of lignin, relative to that in plants, can be explained by a constant, large, non-vascular plant contribution.

The higher ^{13}C content of TOC (Table 2.1) during high discharge could be caused by either input of material with an enriched carbon isotope signal, such as C_4 plant material, or less degraded plant material (Benner et al., 1987). As plant material degrades, the more labile components are preferentially removed, leaving behind the more recalcitrant, and isotopically depleted, lignin pool. Therefore, fresher plant litter has a more positive TOC $\delta^{13}C$ value than degraded material. Therefore, one possible explanation for the observed, more positive $\delta^{13}C$ values in the creek-bank site during high-discharge periods is that it receives an influx of fresh, non-woody, vascular plant material. The high discharge period coincides with, and follows, the dying off period of *S. alterniflora* and the signal may indicate incorporation of newly senescent plant litter into surface sediments. The high discharge period begins in the late fall (Figure 2.2), when *S. alterniflora* is senescent (Gallagher et al., 2005) As the season progressed into the low discharge period, the surface material continues to degrade and age, but new inputs are scarce as *S. alterniflora* is in the growing phase.

Comparing TOC $\delta^{13}C$ values and vanillin $\delta^{13}C$ values (Figure 2.6) supports the observation that carbon dynamics at the creek-bank site are driven by different vascular plant inputs during high and low discharge periods. Although a large variability in the $\delta^{13}C$ values of lignin phenols was observed during lignin degradation (Chapter 3), the $\delta^{13}C$ of vanillin varied minimally (1 – 3‰) with the exception of degradation in seawater, which produced more extensive variability in $\delta^{13}C$ values (~ 7‰; Chapter 3). Assuming that degradation of lignin occurred in sediments, only minor variability in $\delta^{13}C$ values of

vanillin are expected this parameter can relay information on carbon dynamics in this system. As such, the $\delta^{13}C$ values of vanillin indicate that carbon dynamics are driven by differential vascular plant inputs.

Using a linear mixing model:

$$\delta^{13}C_{VAL,sediment} = f_{S.alterniflora}\delta^{13}C_{S.alterniflora} + f_{J.roemarianus}\delta^{13}C_{J.roemarianus} \quad (1)$$

and the assumption that $f_{S.\ alterniflora} + f_{J.\ roemarianus} = 1$, the relative contribution (f) of *S. alterniflora* and *J. roemarianus*, to creek bank sediments, were calculated and both were found to contribute substantially.

The variations in the measured parameters (C/N ratios increase, C/V ratios decrease and TOC $\delta^{13}C$ decrease) during periods of low discharge can be explained by a combination of processes. During periods of high discharge, less-degraded, non-woody, C_4 material is depositied. During the low discharge period, more degraded C_3 material is deposited. This can be explained by the influx of C_4 material in the creeks and the subsequent deposition on the high-marsh, during periods of flooding. This trend reverses during the low discharge period, when the vascular plant material that was deposited during high discharge period is degraded.

4.2.2. High-marsh site

Lignin parameters indicated that organic matter processes at the high-marsh site differ from those at the creek bank site. Temporal variations in S/V and C/V signals suggested differential deposition of plant matter during high and low discharge periods. The high S/V and C/V ratios (Table 2.1) during high discharge correspond to non-woody

angiosperm tissues. The increased OC content during this period illustrates the presence of more degraded, protein-poor material during this period.

In general, different processes may drive the overall sediment dynamics at the high-marsh site, relative to the creek-bank site. Sediments at the high-marsh site had lignin phenol $\delta^{13}C$ values that were generally more enriched in ^{13}C than in *J. roemarianus* (Figure 2.4). The $\delta^{13}C$ values of molecules are observed to increase with degradation (Sun et al., 2004; Liu et al., 2010). However, mixing of C_4 *S. alterniflora* into the sediments could also be responsible for the observed signal. A simple mixing model (Equation 1) was used to test this scenario that a large contribution of *S. alterniflora* was driving carbon dynamics at the high marsh site. When the effect of degradation on isotopic signals of compounds was included (assuming a 2‰ enrichment, based on values measured during sediment degradation (Chapter 3), the mean vanillin $\delta^{13}C$ value of degraded *J. roemarianus* would be ~ -27 ‰. But this enrichment is insufficient to account for the vanillin isotopic value (-25‰) measured in these sediments, indicating additional input of ^{13}C enriched C_4 material. The relative contributions of *S. alterniflora* and *J. roemarianus* to the $\delta^{13}C$ signal of vanillin, using Equation 1, were calculated to be 16% and 83% respectively.

In addition, the TOC $\delta^{13}C$ of high-marsh sediments varied more than that of creek-bank sediments, while the $\delta^{13}C$ signal of vanillin at the high-marsh exhibited less variation (Figure 2.6). The presence of a greater non vascular-plant component at the high-marsh site would result in a greater spread in TOC $\delta^{13}C$ values, but a narrow range of $\delta^{13}C$ values of vanillin, as the non vascular-plant component would not contribute to this pool. While the creek-bank site sediments seem to be dominated by inputs of

vascular plant tissue, the high-marsh site sediments have significant inputs of non-vascular plant origin.

4.2.3. PCA analysis results

PCA analysis results supported the finding that distinctly different carbon dynamics occurred at the creek-bank site and the high-marsh site. The plot of variable loadings of PC1 versus PC2, reflected the correlation between each variable and each PC (Figure 2.7a). PC1 is most negatively loaded on carbon isotope compositions of lignin phenols (loadings < - 0.3; Figure 2.7), and is most positively loaded on C/N ratios (loading ~ 0.3). The more negative loadings of $\delta^{13}C$ values reflected a negative correlation with isotope values and the positive loading of C/N reflected a positive correlation with C/N ratios. Therefore, PC1 separated samples into two groups: samples with high C/N and low $\delta^{13}C$ values (more negative) to the right of the axis, and samples with low C/N and high $\delta^{13}C$ values (more positive). Therefore, variables were separated to define C_4 and C_3 rich organic matter along PC1. Variables were also separated onto PC2, which was based on lignin-rich (below the axis) versus lignin poor (above the axis). The PC scores indicate that the creek bank sediments exhibited more variability (Figure 2.7b), but were predominately composed of C_4 organic matter that was at times lignin-rich and at times lignin-poor. On the other hand, sediments from the high-marsh site clustered closely along PC. These sediments are best described as being composed of C_3 material and lignin poor material.

5. Conclusions

Over the course of the study, the stable carbon isotope compositions of lignin phenols in *S. alterniflora* and *J. roemarianus* varied. The range of variance differed for each compound and for either plant. For each compound, the spread in values was generally greater in *S. alterniflora* than in *J. roemarianus*. In both plants, two compounds, ferulic acid and vanillin, exhibited minor variation. A more precise $\delta^{13}C$ value, indicative of photosynthetic pathway (e.g. C_3 versus C_4) would provide more robust identification of plant material. Vanillin seems to be the a useful biomarker due to its low variability observed in $\delta^{13}C$ values, and the ubiquitous nature of vanillin in all vascular plant structural components. While a more thorough investigation into the variance in $\delta^{13}C$ values of vanillin in other plants is necessary to verify that variations in $\delta^{13}C$ values are sufficiently minor.

Studies on the organic matter cycling in a southeastern Georgia salt marsh environment indicated that the two study sites are dynamic and influenced by different environmental regimes controlling sediment processes. The creek-bank site organic matter is largely of vascular plant origin, with equal contributions from C_3 and C_4 plants. Newly senescent materials are deposited during high discharge periods and as the season progresses into the following low-discharge period, the surface materials continue to age and degrade. The vascular plant material within sediments at the high-marsh site, on the other hand, is predominately composed of lignin from the dominant macrophyte, *J. roemarianus*, with minor inputs of C_4 plant material. It is clear that while the creek-bank site is composed predominately of vascular plant organic matter, the high-marsh site has an ample contribution of non vascular-plant organic matter.

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Table 2.1 Temporal variations of organic carbon (%OC) content, total OC $\delta^{13}C_{OC}$, TN (%), C/N ratios and ID for PCA. The means of each season and of the study period were calculated and standard deviation is reported in parentheses. Statistically significant ($p < 0.05$) differences between seasons are depicted in bold.

Sample Date	PCA ID[a]	%OC		$\delta^{13}C_{OC}$ (‰)		% TN		C/N (atomic)	
Spartina alterniflora									
Nov-05		40.73		-13.5		1.00		47.7	
Dec-05		36.94		-13.1		1.07		40.4	
Jan-06		39.47		-13.2		0.98		47.2	
Feb-06		41.06		-13.5		1.08		44.3	
Mar-06		39.74		-13.5		1.28		36.2	
Apr-06		38.80		-13.3		1.55		29.3	
May-06		39.85		-13.4		0.91		51.1	
Jun-06		39.81		-13.9		1.76		26.4	
Jul-06		38.85		-13.5		1.40		32.4	
Aug-06		40.12		-13.7		1.36		34.4	
Sep-06		40.32		-14.3		1.68		28.0	
Oct-06		42.86		-13.9		0.78		64.1	
Nov-06		40.65		-13.8		0.77		61.6	
Feb-07		38.41		-13.8		0.89		50.3	
Mar-07		38.33		-13.8		1.00		44.7	
Apr-07		37.42		-13.9		1.21		36.1	
Fall/Winter *(n=9)*		39.80	(1.76)	-13.6	(0.3)	**0.98**	**(0.16)**	**48.5**	**(9.1)**
Spring/Summer *(n=7)*		39.31	(1.02)	-13.7	(0.3)	**1.41**	**(0.29)**	**33.9**	**(8.3)**
Study *(n=16)*		39.58	(1.46)	-13.6	(0.3)	1.17	(0.31)	42.1	(11.3)
Creek Bank Sediments									
Nov-05	C1	1.84		-22.6		0.12		18.2	
Dec-05	C2	2.69		-20.8		0.23		13.9	
Jan-06	C3	2.90		-20.7		0.27		12.3	
Feb-06	C4	3.54		-20.5		0.34		12.2	
Mar-06	C5	4.53		-20.3		0.40		13.2	
Apr-06	C6	2.72		-21.0		0.24		13.0	
May-06	C7	3.69		-21.3		0.32		13.5	
Jun-06	C8	1.83		-21.2		0.13		16.1	
Jul-06	C9	1.60		-21.2		0.13		14.1	
Aug-06	C10	1.35		-20.9		0.23		6.8	
Sep-06	C11	1.76		-21.2		0.12		17.1	
Oct-06	C12	1.46		-20.3		0.12		14.7	
Nov-06	C13	3.37		-21.0		0.24		16.4	
Dec-06	C14	2.92		-21.0		0.17		20.0	
Jan-07	C15	2.91		-20.3		0.21		16.5	

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Table 2.1 (continued)

Sample Date	PCA ID[a]	%OC	$\delta^{13}C_{OC}$ (‰)	% TN	C/N (atomic)
Feb-07	C16	1.82	-21.1	0.24	9.0
Mar-07	C17	1.52	-19.6	0.21	8.6
Apr-07	C18	1.16	-20.6	0.13	10.7
May-07	C19	3.77	-22.1	0.18	24.6
Low Discharge *(n=10)*		2.36 (0.97)	**-21.3 (0.6)**	**0.18 (0.07)**	**16.1 (4.6)**
High Discharge *(n=9)*		2.64 (1.04)	**-20.5 (0.5)**	**0.25 (0.08)**	**12.1 (2.5)**
Study *(n=19)*		2.49 (0.98)	-20.9 (0.7)	0.21 (0.08)	14.3 (4.2)
Juncus roemarianus					
May-06		44.76	-26.8	0.97	53.8
Jun-06		44.11	-26.8	1.23	41.8
Jul-06		45.11	-26.5	0.83	63.4
Aug-06		45.68	-27.3	0.89	59.9
Sep-06		41.72	-27.1	0.73	66.6
Oct-06		46.68	-26.8	0.77	70.7
Nov-06		46.35	-26.7	0.78	69.3
Jan-07		45.72	-27.0	0.79	67.5
Apr-07		43.91	-24.8	0.63	81.3
May-07		44.50	-26.0	0.72	72.1
Fall/Winter *(n=3)*		**46.25 (0.49)**	-26.8 (0.1)	0.78 (0.01)	69.2 (1.6)
Spring/Summer *(n=6)*		**44.26 (1.27)**	-26.5 (0.8)	0.86 (0.20)	62.7 (12.7)
Study l *(n=9)*		44.85 (1.43)	-26.6 (0.7)	0.83 (0.17)	64.6 (10.9)
High Marsh Sediments					
May-06	H1	0.91	-21.0	0.07	14.9
Jun-06	H2	0.69	-23.6	0.05	15.6
Jul-06	H3	1.24	-23.3	0.06	23.2
Aug-06	H4	0.82	-24.5	0.06	16.8
Sep-06	H5	0.73	-22.9	0.06	13.4
Oct-06	H6	1.01	-24.3	0.05	22.3
Nov-06	H7	0.67	-23.3	0.05	16.2
Dec-06	H8	0.51	-23.1	0.04	16.6
Jan-07	H9	0.66	-20.8	0.04	18.5
Feb-07	H10	1.39	-23.1	0.06	28.1
Mar-07	H11	2.09	-20.9	0.10	24.8
May-07	H12	0.56	-21.3	0.03	21.5
Low Discharge *(n=9)*		**0.79 (0.23)**	-23.0 (1.2)	0.05 (0.01)	17.8 (3.5)
High Discharge *(n=3)*		**1.38 (0.71)**	-21.6 (1.3)	0.07 (0.03)	23.8 (4.9)
Study *(n=12)*		0.94 (0.45)	-22.7 (1.3)	0.06 (0.02)	19.3 (4.5)

Table 2.2 Lignin phenol content (VAL-vanillin, VON-acetovanillone, VAD-vanillic acid, SAL-syringaldehyde, SON-acetosyringone, SAD-syringic acid, CAD-p-coumaric acid, FAD-ferulic acid), Λ_6 (sum of vanillyl and syringyl phenols, normalized to 100 mg OC), and Λ_8 (sum of syringyl, vanillyl, and cinnamyl phenols, normalized to 100 mg OC). The means of each season and of the study period were calculated and standard deviation is reported in parentheses. Statistically significant ($p < 0.05$) differences between seasons are depicted in bold.

Sample Date	VAL	VON	VAD	SAL	SON	SAD	CAD	FAD	Λ_6	Λ_8
Spartina alterniflora										
Nov-05	2.05	0.31	0.19	2.10	0.78	0.16	1.08	1.50	5.58	8.17
Dec-05	2.38	0.49	0.42	1.15	1.07	0.46	0.97	1.99	5.97	8.92
Jan-06	2.25	0.44	0.41	1.18	1.11	0.55	1.06	2.65	5.94	9.65
Feb-06	2.54	0.66	0.94	1.44	1.43	1.29	1.09	3.27	8.30	12.66
Mar-06	1.86	0.39	0.29	1.14	0.89	0.36	0.75	0.79	4.93	6.47
Apr-06	2.02	0.37	0.66	0.76	0.82	0.71	0.74	1.23	5.34	7.32
May-06	2.44	0.46	0.43	1.37	0.83	0.36	0.81	0.77	5.90	7.48
Jun-06	2.19	0.41	0.45	0.81	0.94	0.57	0.78	1.92	5.36	8.06
Jul-06	2.39	0.58	0.31	1.38	1.06	0.31	0.84	1.37	6.04	8.25
Aug-06	2.79	0.51	0.47	1.51	1.29	0.26	2.39	4.07	6.82	13.28
Sep-06	2.27	0.46	0.51	1.17	1.16	0.21	1.78	3.17	5.77	10.72
Oct-06	2.80	0.50	0.24	1.31	1.07	0.35	0.79	1.72	6.27	8.78
Nov-06	2.89	0.44	0.33	0.98	0.83	0.46	0.73	1.62	5.94	8.30
Feb-07	2.88	0.51	0.52	1.70	1.20	0.50	1.03	1.68	7.30	10.02
Mar-07	2.49	0.54	0.48	0.99	1.27	0.62	0.98	2.51	6.40	9.89
Apr-07	2.81	0.35	0.51	0.80	0.68	0.28	0.66	2.03	5.44	8.13
Fall/Winter *(n=9)*	2.46 (0.36)	0.48 (0.10)	0.42 (0.22)	1.33 (0.37)	1.07 (0.21)	0.53 (0.32)	0.94 (0.15)	1.97 (0.74)	6.29 (0.99)	9.21 (1.69)
Spring/Summer *(n=7)*	2.41 (0.30)	0.45 (0.08)	0.48 (0.10)	1.12 (0.32)	0.97 (0.21)	0.39 (0.19)	1.14 (0.67)	2.08 (1.16)	5.81 (0.52)	9.03 (2.18)
Study *(n=16)*	2.44 (0.33)	0.46 (0.09)	0.45 (0.18)	1.24 (0.35)	1.03 (0.21)	0.47 (0.27)	1.03 (0.45)	2.02 (0.91)	6.08 (0.83)	9.13 (1.86)
Creek Bank Sediments										
Nov-05	2.64	0.53	0.37	3.07	0.67	0.33	0.54	0.42	7.60	8.55
Dec-05	1.42	0.53	0.38	1.26	0.61	0.33	0.44	0.40	4.53	5.38
Jan-06	0.85	0.27	0.24	0.73	0.39	0.22	0.36	0.54	2.69	3.59
Feb-06	1.13	0.35	0.38	0.98	0.43	0.39	0.47	0.75	3.66	4.88
Mar-06	1.03	0.36	0.25	0.85	0.34	0.22	0.44	0.55	3.05	4.04

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Table 2.2 *(continued)*

Sample Date	VAL	VON	VAD	SAL	SON	SAD	CAD	FAD	Λ_6	Λ_8
Apr-06	1.21	0.37	0.32	0.97	0.36	0.29	0.25	0.24	3.52	4.01
May-06	0.94	0.27	0.23	0.87	0.28	0.22	0.26	0.23	2.81	3.30
Jun-06	1.44	0.36	0.36	1.12	0.40	0.26	0.26	0.15	3.94	4.35
Jul-06	1.46	0.43	0.41	1.32	0.49	0.37	0.29	0.20	4.47	4.96
Aug-06	1.86	0.45	0.67	1.45	0.67	0.72	0.42	0.45	5.82	6.69
Sep-06	1.17	0.29	0.41	0.91	0.39	0.37	0.34	0.23	3.54	4.11
Oct-06	1.71	0.40	0.55	1.51	0.64	0.59	0.56	0.32	5.39	6.27
Nov-06	1.37	0.31	0.52	0.84	0.40	0.46	0.39	0.41	3.90	4.69
Dec-06	1.26	0.46	0.25	1.08	0.50	0.24	0.24	0.28	3.80	4.31
Jan-07	1.29	0.39	0.65	0.98	0.74	0.54	0.41	0.79	4.59	5.80
Feb-07	2.36	0.50	1.20	1.02	0.68	0.19	0.36	1.03	5.96	7.35
Mar-07	2.80	0.39	1.49	1.08	0.58	0.49	0.48	1.68	6.84	8.99
Apr-07	1.63	0.52	1.04	1.45	0.94	0.45	0.64	0.98	6.03	7.65
May-07	3.91	0.39	1.34	0.69	0.62	0.28	0.26	0.62	7.22	8.10
Low Discharge *(n=10)*	1.78 (0.88)	0.39 (0.08)	0.51 (0.32)	1.28 (0.68)	0.51 (0.14)	0.38 (0.16)	0.36 (0.12)	**0.33 (0.14)**	4.85 (1.61)	5.53 (1.78)
High Discharge *(n=9)*	1.52 (0.65)	0.41 (0.09)	0.66 (0.47)	1.04 (0.21)	0.56 (0.2)	0.35 (0.13)	0.43 (0.1)	**0.77 (0.43)**	4.54 (1.46)	5.74 (1.88)
Study *(n=19)*	1.66 (0.77)	0.40 (0.08)	0.58 (0.39)	1.17 (0.52)	0.53 (0.17)	0.37 (0.14)	0.39 (0.12)	0.54 (0.38)	4.70 (1.5)	5.63 (1.78)
Juncus roemarianus										
May-06	2.34	0.46	0.36	2.81	0.70	0.47	1.35	0.63	7.14	9.12
Jun-06	2.54	0.46	0.54	1.95	0.75	0.61	1.07	0.66	6.84	8.58
Jul-06	2.55	0.28	0.76	1.17	0.83	1.31	1.33	1.27	6.90	9.49
Aug-06	2.35	0.36	0.22	3.70	0.68	0.30	0.88	0.56	7.62	9.06
Sep-06	2.73	0.47	0.46	5.14	1.00	0.76	1.41	0.97	10.56	12.94

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Table 2.2 *(continued)*

Sample Date	VAL	VON	VAD	SAL	SON	SAD	CAD	FAD	Λ_6	Λ_8
Oct-06	2.40	0.40	0.28	3.75	0.76	0.46	1.36	0.81	8.05	10.22
Nov-06	2.23	0.43	0.40	4.14	0.93	0.80	1.54	0.76	8.93	11.23
Jan-07	2.55	0.38	0.51	4.62	1.14	0.81	1.88	1.65	10.01	13.54
Apr-07	2.99	0.46	0.46	4.90	1.02	0.73	1.61	1.18	10.56	13.35
May-07	2.67	0.47	0.43	5.51	1.15	0.60		0.97	10.84	
Fall/Winter *(n=3)*	2.39 (0.16)	0.40 (0.02)	0.40 (0.12)	4.17 (0.43)	0.94 (0.19)	0.69 (0.20)	1.59 (0.26)	1.08 (0.50)	9.00 (0.98)	11.66 (1.71)
Spring/Summer *(n=6)*	2.57 (0.23)	0.42 (0.08)	0.46 (0.16)	3.34 (1.68)	0.86 (0.18)	0.67 (0.32)	1.25 (0.26)	0.88 (0.28)	8.32 (1.90)	9.92 (2.13)
Study *(n=9)*	2.54 (0.22)	0.42 (0.06)	0.44 (0.15)	3.77 (1.42)	0.90 (0.18)	0.69 (0.28)	1.38 (0.29)	0.95 (0.34)	8.74 (1.63)	10.83 (1.99)
High Marsh Sediments										
May-06	1.39	0.278	0.46	0.729	0.218	0.297	0.306	0.188	3.37	3.87
Jun-06	1.72	0.248	0.44	0.944	0.275	0.211	0.138	0.042	3.84	4.02
Jul-06	1.92	0.266	0.51	0.964	0.295	0.335	0.313	0.210	4.30	4.82
Aug-06	1.58	0.382	0.42	1.871	0.511	0.367	0.304	0.207	5.56	5.64
Sep-06	0.99	0.199	0.28	0.242	0.253	0.143	0.148	0.201	2.11	2.46
Oct-06	1.03	0.181	0.46	1.135	0.424	0.390	0.444	0.233	3.61	4.29
Nov-06	0.75	0.192	0.31	0.798	0.232	0.257	0.169	0.070	2.54	2.78
Dec-06	1.26	0.421	0.64	1.415	0.737	0.535	0.354	0.278	6.48	5.64
Jan-07	0.82	0.224	0.23	0.961	0.324	0.267	0.199	0.405	2.82	3.43
Feb-07	0.61	0.179	0.25	0.765	0.276	0.229	0.159	0.144	2.30	2.61
Mar-07	0.53	0.164	0.17	0.753	0.240	0.111	0.179	0.285	1.97	2.43
May-07	1.17	0.226	0.48	0.880	0.485	0.181	0.176	0.242	3.42	3.84
Low Discharge *(n=9)*	**1.31 (0.38)**	0.27 (0.08)	**0.44 (0.11)**	1.00 (0.46)	0.38 (0.17)	0.30 (0.12)	0.26 (0.11)	0.19 (0.08)	3.91 (1.38)	4.15 (1.11)
High Discharge *(n=3)*	**0.65 (0.15)**	0.19 (0.03)	**0.22 (0.04)**	0.83 (0.12)	0.28 (0.04)	0.20 (0.08)	0.18 (0.02)	0.28 (0.13)	2.37 (0.43)	2.82 (0.53)
Study *(n=12)*	1.15 (0.44)	0.25 (0.08)	0.39 (0.14)	0.95 (0.40)	0.36 (0.16)	0.28 (0.12)	0.24 (0.10)	0.21 (0.10)	3.53 (1.38)	3.82 (1.14)

Table 2.3 Lignin phenol parameters: (Ad/Al)v – ratio of vanillic acid to vanillin; $(Ad/Al)_S$ - ratio of syringic acid to syringyl; S/V - ratio of syringyl phenols to vanillyl phenols; C/V - ratio of cinnamyl phenols to vanillyl phenols. Statistically significant ($p < 0.05$) differences between seasons are depicted in bold.

Sample Date	$(Ad/Al)_V$	$(Ad/Al)_S$	S/V	C/V
Spartina alterniflora				
Nov-05	0.09	0.08	1.2	1.0
Dec-05	0.17	0.40	0.81	0.90
Jan-06	0.18	0.47	0.92	1.2
Feb-06	0.37	0.90	1.0	1.1
Mar-06	0.16	0.31	0.94	0.60
Apr-06	0.32	0.94	0.75	0.65
May-06	0.18	0.27	0.77	0.47
Jun-06	0.21	0.70	0.76	0.88
Jul-06	0.13	0.23	0.84	0.67
Aug-06	0.17	0.17	0.81	1.7
Sep-06	0.22	0.18	0.78	1.5
Oct-06	0.09	0.27	0.77	0.71
Nov-06	0.12	0.47	0.62	0.64
Feb-07	0.18	0.29	0.87	0.69
Mar-07	0.19	0.62	0.82	1.00
Apr-07	0.18	0.35	0.48	0.73
Fall/Winter *(n=9)*	0.17 (0.08)	0.42 (0.24)	0.88 (0.16)	0.87 (0.21)
Spring/Summer *(n=7)*	0.20 (0.06)	0.40 (0.30)	0.74 (0.12)	0.95 (0.48)
Study *(n=16)*	0.19 (0.07)	0.41 (0.25)	0.82 (0.16)	0.90 (0.34)
Creek Bank Sediments				
Nov-05	0.14	0.11	1.1	0.27
Dec-05	0.27	0.26	0.95	0.36
Jan-06	0.28	0.30	1.00	0.67
Feb-06	0.34	0.39	0.97	0.65
Mar-06	0.24	0.26	0.86	0.61
Apr-06	0.27	0.29	0.85	0.26
May-06	0.24	0.26	0.96	0.34
Jun-06	0.25	0.23	0.82	0.19
Jul-06	0.28	0.28	0.94	0.21
Aug-06	0.36	0.50	0.95	0.29
Sep-06	0.35	0.40	0.89	0.31
Oct-06	0.32	0.39	1.0	0.33
Nov-06	0.38	0.54	0.77	0.36
Dec-06	0.20	0.22	0.92	0.26
Jan-07	0.50	0.55	0.97	0.52

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Table 2.3 *(continued)*

Sample Date	$(Ad/Al)_V$	$(Ad/Al)_S$	S/V	C/V
Feb-07	0.51	0.19	0.47	0.34
Mar-07	0.53	0.45	0.46	0.46
Apr-07	0.64	0.31	0.89	0.51
May-07	0.34	0.40	0.28	0.16
Low Discharge *(n=10)*	0.29 (0.08)	0.33 (0.14)	0.87 (0.23)	**0.27 (0.07)**
High Discharge *(n=9)*	0.40 (0.15)	0.33 (0.11)	0.82 (0.21)	**0.49 (0.14)**
Study *(n=19)*	0.34 (0.13)	0.33 (0.12)	0.85 (0.22)	0.37 (0.15)
Juncus roemarianus				
May-06	0.15	0.17	1.3	0.62
Jun-06	0.21	0.31	0.94	0.49
Jul-06	0.30	4.9	0.92	0.72
Aug-06	0.09	0.08	1.6	0.49
Sep-06	0.17	0.15	1.9	0.65
Oct-06	0.12	0.12	1.6	0.71
Nov-06	0.18	0.19	1.9	0.75
Jan-07	0.20	0.18	1.9	1.0
Apr-07	0.15	0.15	1.7	0.71
May-07	0.16	0.11	2.0	0.27
Fall/Winter *(n=3)*	0.16 (0.04)	0.16 (0.04)	1.8 (0.17)	0.83 (0.17)
Spring/Summer *(n=6)*	0.18 (0.06)	0.83 (1.8)	1.5 (0.44)	0.57 (0.16)
Study *(n=9)*	0.17 (0.06)	0.63 (1.5)	1.6 (0.41)	0.64 (0.20)
High Marsh Sediments				
May-06	0.33	0.41	0.58	0.23
Jun-06	0.26	0.22	0.59	0.07
Jul-06	0.27	0.35	0.59	0.19
Aug-06	0.26	0.20	1.2	0.21
Sep-06	0.28	0.59	0.43	0.24
Oct-06	0.45	0.34	1.2	0.41
Nov-06	0.42	0.32	1.0	0.19
Dec-06	0.51	0.38	1.2	0.27
Jan-07	0.28	0.28	1.2	0.48
Feb-07	0.41	0.30	1.2	0.29
Mar-07	0.33	0.15	1.3	0.53
May-07	0.41	0.21	0.82	0.22
Low Discharge *(n=9)*	0.35 (0.09)	0.33 (0.12)	**0.84 (0.30)**	**0.23 (0.09)**
High Discharge *(n=3)*	0.34 (0.06)	0.24 (0.08)	**1.2 (0.03)**	**0.43 (0.13)**
Study *(n=12)*	0.35 (0.08)	0.31 (0.12)	0.94 (0.31)	0.28 (0.13)

Figure 2.1 Map of study site in southeastern coastal Georgia, USA. *S. alterniflora* and creek bank sediments were collected from the creek-bank site (A) and *J. roemarianus* and high-marsh sediments were collected from the high-marsh site (B).

Figure 2.2 Physical parameters measured at Hudson Creek near the creek bank site. Data provided by DiIorio and the GCE-LTER.

Figure 2.3 Box plots depicting the median (horizontal lines inside boxes) interquartile range (box ends), and the extreme data limits (the whiskers) in $\delta^{13}C$ values for the lignin phenols. Box plots depicting *S. alterniflora* data are in gray, whereas those for *J. roemarianus* are white. Dashed lines depict the $\delta^{13}C$ values of TOC in *S. alterniflora* (top) and *J. roemarianus* (bottom), and standard deviations in TOC $\delta^{13}C$ are delineated in smaller dotted lines. The lines delineating the standard deviation of *S. alterniflora* TOC are too close to distinguish. VAL- vanillin, AVO - acetovanillone, SAL - syringaldehyde, ASO - acetosyringone, VAD - vanillic acid, SAD - syringic acid, CAD - p-coumaric acid, FAD - ferulic acid.

Figure 2.4 Temporal variation in lignin-phenol compound-specific $\delta^{13}C$ values in: (**a**) *S. alterniflora* (gray circles), creek-bank sediments (black circles), and (**b**) *J. roemarianus* (gray squares), and high-marsh sediments (black squares). Months are identified by number.

Figure 2.5 Lignin parameter plot showing ratio of cinnamyl to vanillyl (C/V) and syringyl to vanillyl (S/V) ratios for *S. alterniflora* (open circles), creek-bank sediments (gray circles), *J. roemarianus* (open squares), and high-marsh sediments (gray squares). The boxed regions represent the compositional ranges for angiosperm woody tissue,

angiosperm non-woody tissue, gymnosperm woody tissue, and gymnosperm non-woody tissue (Hedges and Mann, 1979a).

Figure 2.6 Source partitioning plot depicting the vanillin $\delta^{13}C$ values and TOC $\delta^{13}C$ values of *S. alterniflora* (open circles), creek-bank sediments (gray circles), *J. roemarianus* (open squares), and high-marsh sediments (black squares). The boxed regions represent the compositional ranges for the different sample types. The hatched box represents the ranges in values for *J. roemarianus*; the gray outlined box represents the range in values for high-marsh sediments; the black outlined box represents the range for creek-bank sediments; the dotted box represents the range in values for *S. alterniflora.*

Figure 2.7 PC loadings plot (a) of lignin-phenol compositions and isotope values, C/N ratios, TOC $\delta^{13}C$, and TN $\delta^{15}N$, and PC scores (b) for sediment creek-bank and high-marsh samples.

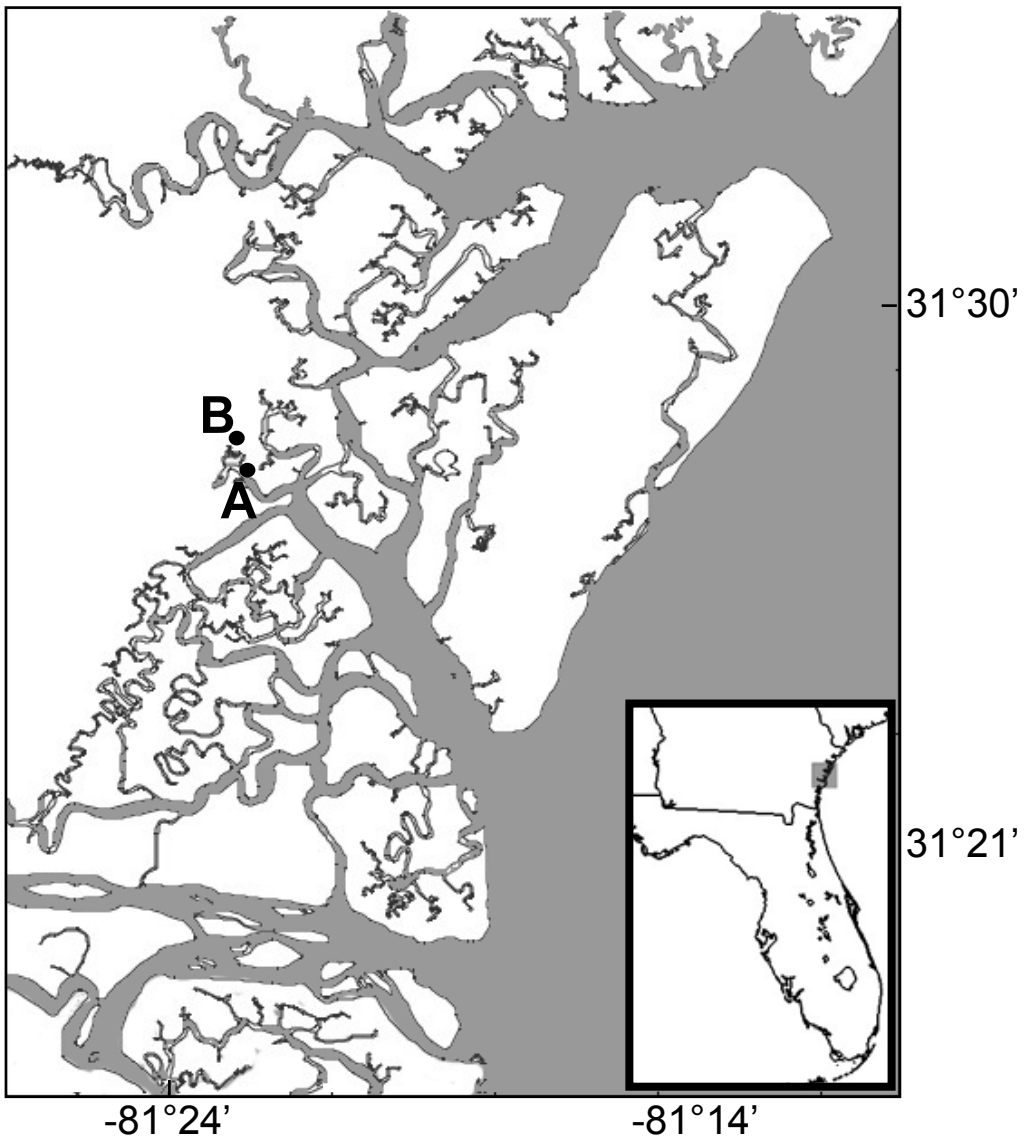

Figure 2.1

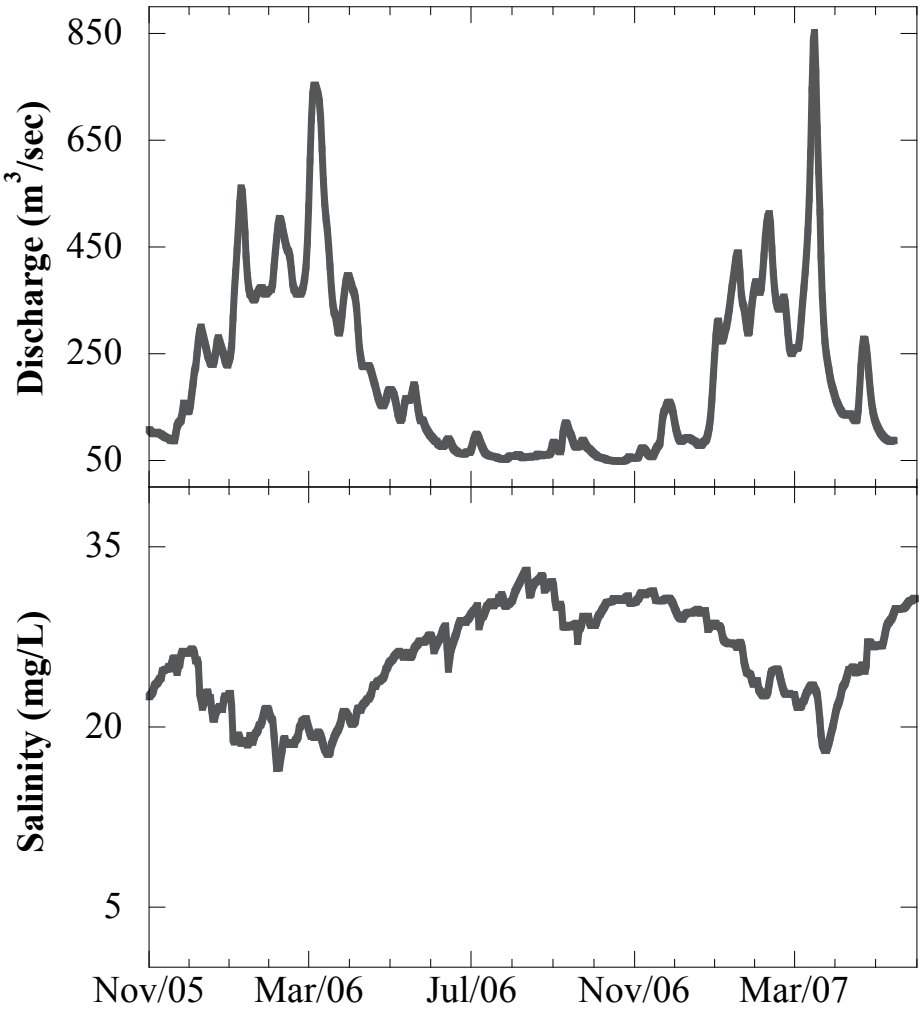
850
650
450
250
50
Discharge (m3/sec)
35
20
5
Salinity (mg/L)
Nov/05
Mar/06
Jul/06
Nov/06
Mar/07

Figure 2.2

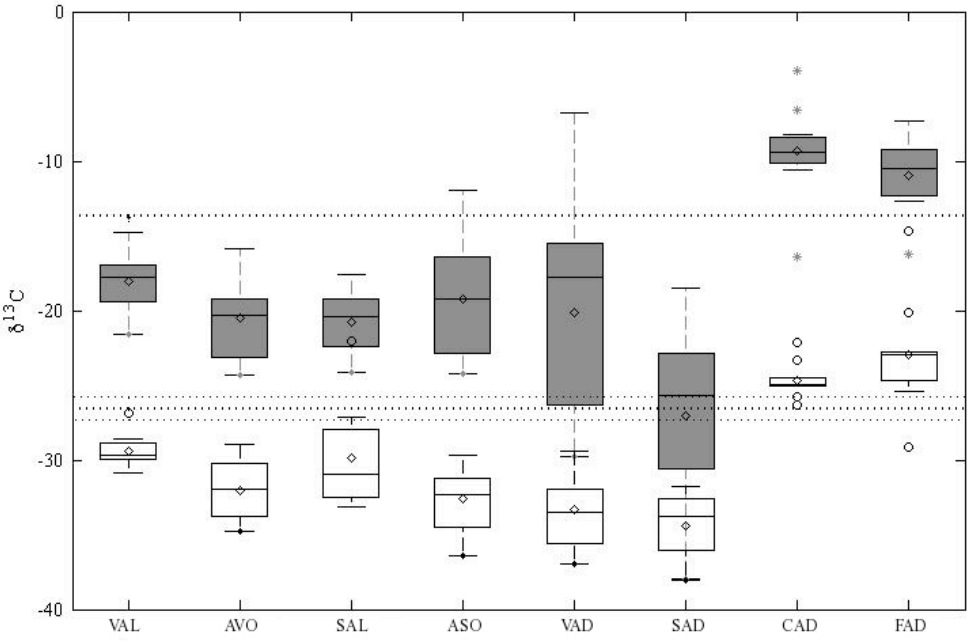

Figure 2.3

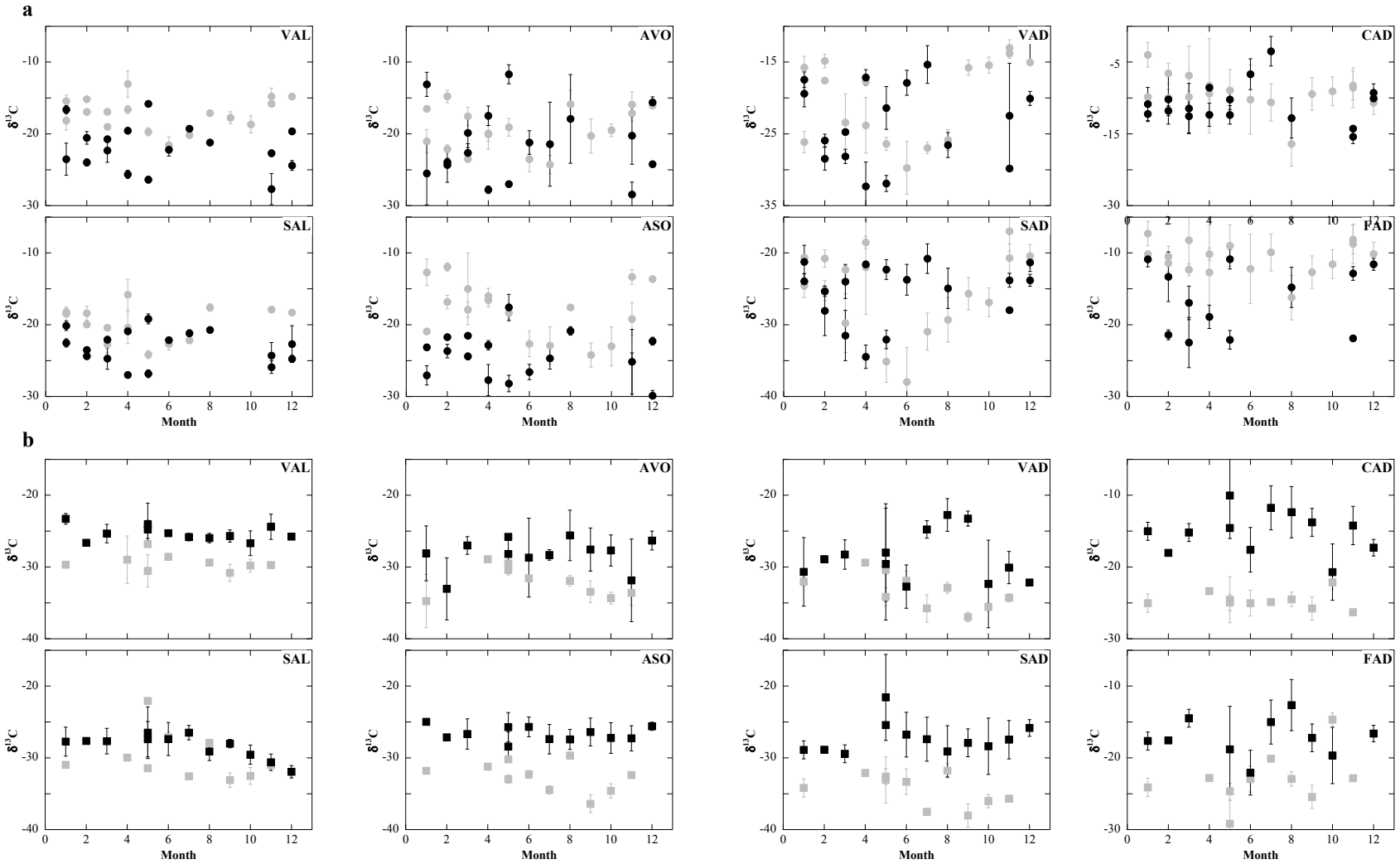
a
VAL
AVO
VAD
CAD
SAL
ASO
SAD
FAD
δ13C
Month
b
VAL
AVO
VAD
CAD
SAL
ASO
SAD
FAD
δ13C
Month

Figure 2.4

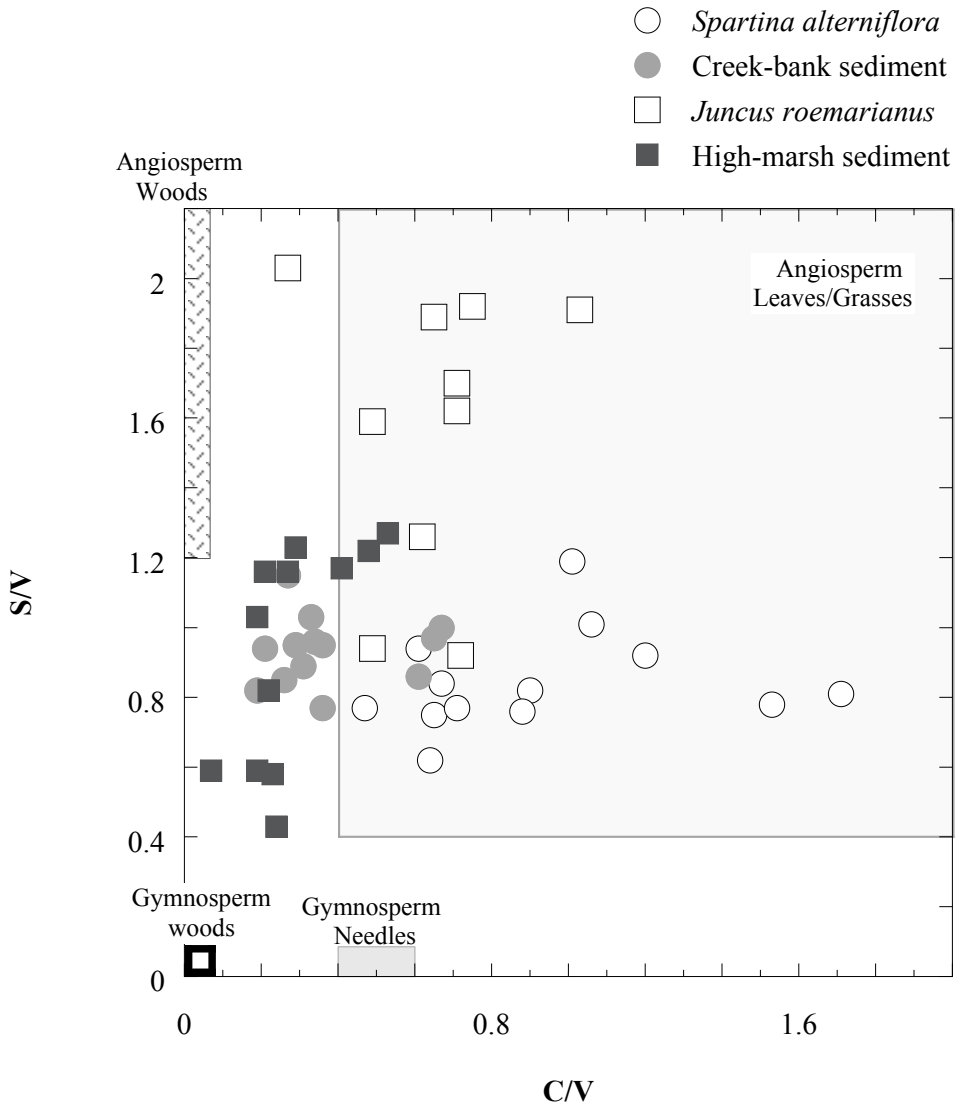
Spartina alterniflora
Creek-bank sediment
Juncus roemarianus
High-marsh sediment
Angiosperm Woods
Angiosperm Leaves/Grasses
Gymnosperm woods
Gymnosperm Needles
S/V
C/V
0
0.4
0.8
1.2
1.6
2
0
0.8
1.6

Figure 2.5

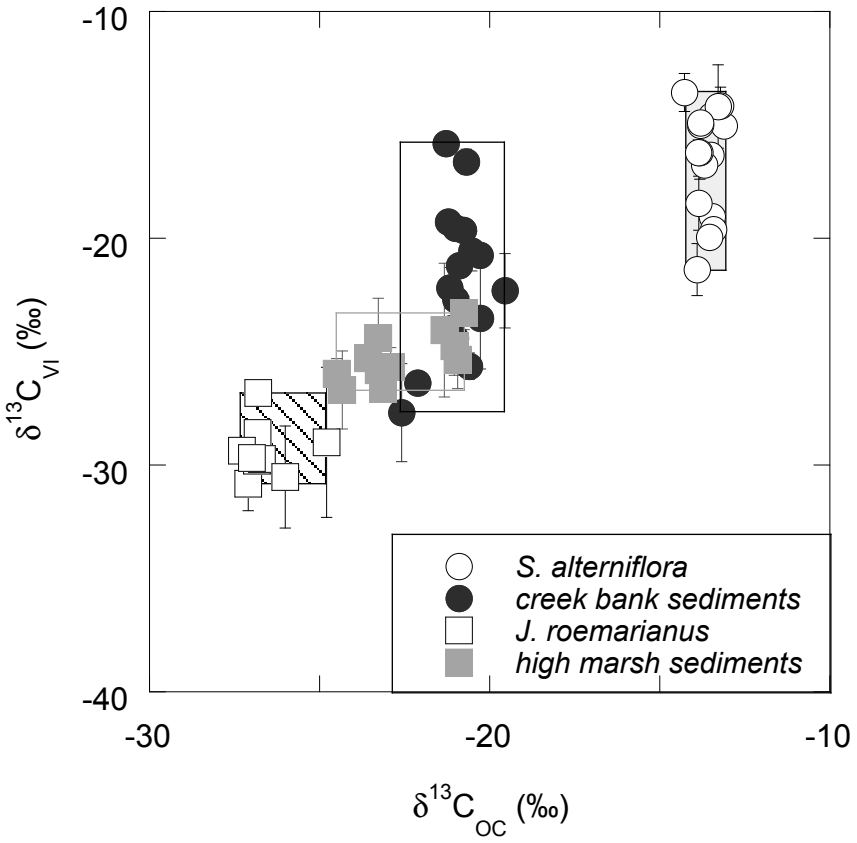
-10
-20
-30
-40
-30
-20
-10
δ13CVI (‰)
δ13COC (‰)
S. alterniflora
creek bank sediments
J. roemarianus
high marsh sediments

Figure 2.6

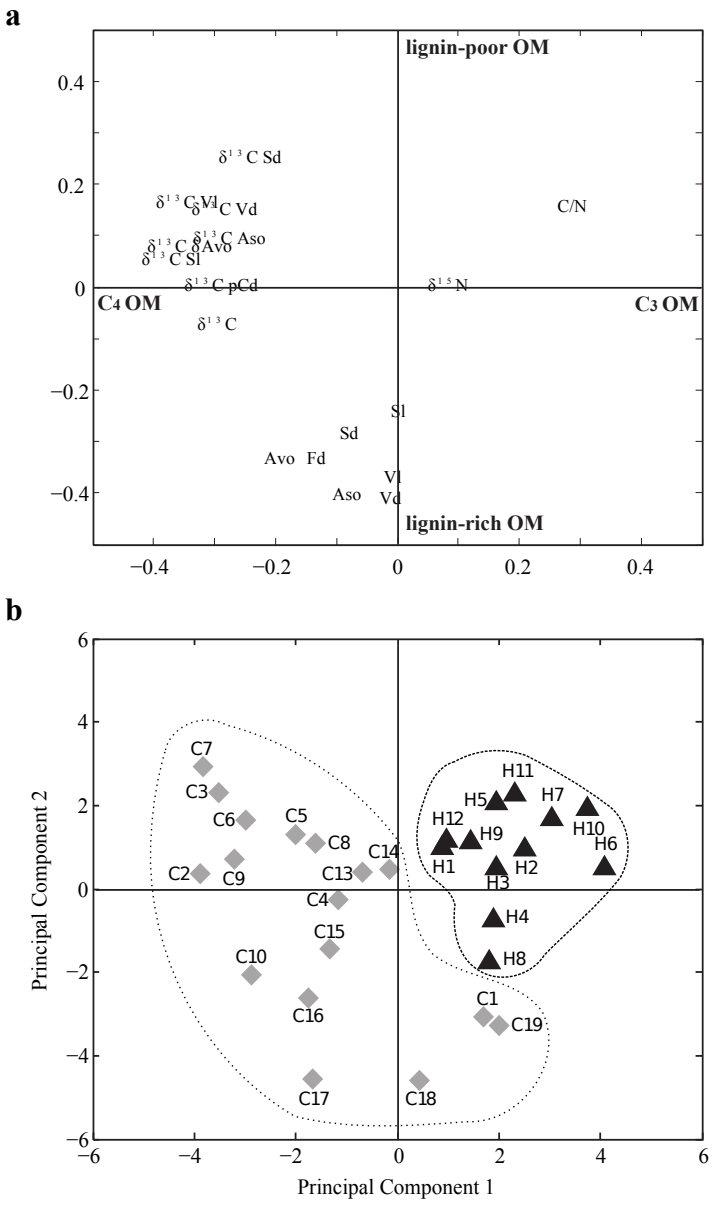
a
lignin-poor OM
C/N
δ15N
C4 OM
C3 OM
δ13C
δ13C Sd
δ13C Vl
δ13C Vd
δ13C Aso
δ13C Avo
δ13C Sl
δ13C pCd
Sl
Sd
Avo
Fd
Vl
Aso
Vd
lignin-rich OM
b
Principal Component 2
Principal Component 1
C7
C3
C6
C5
C8
C14
C2
C9
C13
C4
C15
C10
C16
C17
C18
C1
C19
H11
H5
H7
H12
H9
H10
H6
H1
H2
H3
H4
H8

Figure 2.7

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CHAPTER 3

DEGRADATION OF MARSH PLANT-DERIVED LIGNINS AND THE EFFECT ON COMPOUND-SPECIFIC ISOTOPIC SIGNALS [2]

[2] Loftis, K. M., M-Y. Sun. To be submitted to Organic Geochemistry.

Abstract

Compound-specific isotopic signals ($\delta^{13}C$) of terrestrial plant-derived lignin biomarkers have been applied to distinguish organic carbon inputs from C3 and C4 sources in coastal marine environments. However, it is unclear how the degradation processes of organic matter affect these isotopic signals. This study aims to assess the impacts of decomposition of plant material on the geochemical characteristics including molecular isotopic signals. Two plants, *Spartina alterniflora* and *Juncus roemarianus*, were selected as model organic materials due to their distinct isotopic characteristics (C3 versus C4) and the prevalence in salt marshes of the Southeastern United States. The leaves of these two marsh plants were separately incubated under three different environmental conditions: in constantly oxic seawater, in surface (0-5 cm) and subsurface (>10 cm) sediments of a salt marsh in Sapelo Island, Georgia, USA. The plant leaf materials were incubated for one year and the changes in bulk tissue elemental content (e.g. organic carbon and total nitrogen), bulk tissue $\delta^{13}C$ values, lignin content (Λ_6), lignin degradation parameters (e.g. [(Ad/Al)] ratios and [P/(S+V)] ratios), and compound-specific $\delta^{13}C$ of lignin monomers were monitored over the course of incubation.

Our experimental results showed that the extent of decomposition varied with plant type and environment, with the greatest loss of organic carbon (OC) occurring in the aqueous system for both *S. alterniflora* and *J. roemarianus*. Changes in bulk tissue $\delta^{13}C$ values were also dependent on plant type and environment, with greater changes occurring for *S. alterniflora* than *J. roemarianus*, and in the aqueous system relative to the sediments. The [(Ad/Al)] ratios of *S. alterniflora* and *J. roemarianus* increased when

incubated in oxic seawater, suggesting that both plants experienced side-chain oxidation during decomposition in seawater. During the degradation of *J. roemarianus* in seawater, the [P/(V+S)] ratios decreased, indicating that demethylation, along with side-chain oxidation, was an important process for *J. roemarianus* decomposition. The [P/(V+S)] ratio for *S. alterniflora* in this system could not be interpreted because a non-lignin source of *p*-hydroxy phenols (P) was present so that [P/(V+S)] was not a measure of changes to lignin molecule.

The $\delta^{13}C$ values of lignin phenols from *S. alterniflora* consistently decreased, with depletions ranging from 5-12‰, during seawater degradation. The $\delta^{13}C$ values of *S. alterniflora* degraded in sediments did not vary significantly. Differences observed in the lignin $\delta^{13}C$ values of *S. alterniflora* in these environments may result from differences in the lignin degradation pathway employed. $\delta^{13}C$ values of lignin phenols in *J. roemarianus* did not show a clear increasing or decreasing pattern during seawater decomposition. Three pathways of lignin degradation were evident and the influence of all these pathways seemingly complicated the lignin isotope dynamics. In surface sediments, where lignin in *J. roemarianus* underwent side-chain oxidation and aromatic ring cleavage, the trends in lignin isotope composition resembled that of seawater degradation.

1. Introduction

Lignin is exclusively produced in land ecosystems and is unique to vascular plants and virtually absent in other organisms (Lewis and Yamamoto, 1990). CuO oxidation of plant tissues, sediments, dissolved organic matter, and particulate organic matter yields

monomeric and dimeric lignin-phenols that, upon derivatization, can be analyzed by gas chromatography (Hedges and Ertel, 1982). Lignin $\delta^{13}C$ analysis is utilized to overcome the complexity of total organic carbon isotope analysis for natural bulk samples derived from mixed sources. Compound-specific isotopic analysis of lignin in sediments, and other complex mixtures, yields a more unique parameter for identifying and quantifying the vascular plant component in mixtures. Combining information on the lignin-phenol chemical composition and $\delta^{13}C$ value of the lignin oxidation products, provides the ability to distinguish vascular plant organic matter and determine the relative contribution of C_3 and C_4 vascular plants. Particularly, the $\delta^{13}C$ values of lignin derivatives in sediments were used to distinguish C_3 and C_4 vascular plant inputs from land into the Gulf of Mexico (Goñi and Montgomery, 2000).

Lignin is subject to changes during organic matter degradation and the relative composition of lignin phenols in a degraded sample differs from that of the undegraded material (Hedges et al., 1988; Benner et al., 1991; Opsahl and Benner, 1995). Studies of other biomarkers revealed microbial degradation can also result in changes to the isotopic composition of biomolecules (Macko et al., 1994; Meckenstock et al., 1999; Teece et al., 1999; Sun et al., 2004). The effect of microbiological transformations on organic matter is complex, and the extent and the direction of isotopic alteration are dependent on the molecular structures, cellular and matrix associations, and environmental conditions.

To gain a better understanding of the effects of degradation processes on lignin compositions and their compound-specific isotope signals, we conducted a series of experiments to separately incubate *S. alterniflora* and *J. roemarianus* leaves under three different environmental conditions for one year: (1) in oxic seawater in the dark, (2) in

surface (0-5 cm) sediment in a tidal creek on Sapelo Island, GA, and (3) buried and incubated in subsurface (> 10 cm) sediments of the same creek site. In this study, we are focusing on: (1) how fast do the lignins from different plants degrade under different environmental conditions? (2) what changes occur for lignin compositions and associated indices during degradation? and (3) how much are isotopic signals of lignin-derived phenol biomarkers altered during degradation? We followed the changes in bulk parameters such as total organic carbon and total nitrogen contents, bulk $\delta^{13}C$ values, lignin content (Λ_6), lignin degradation parameters (e.g. [(Ad/Al)] ratios and [P/(S+V)] ratios), and compound-specific $\delta^{13}C$ of lignin monomers. Based on the results from incubation experiments, we explored the possible degradation mechanisms of lignins and their impacts on chemical/isotopic signals in natural systems.

2. Materials and Methods

2.1. Sample preparation and incubation

S. alterniflora (C_4 plant) and *J. roemarianus* (C_3 plant) leaves were incubated in three different environmental conditions over one year to examine the effect of decomposition on the lignin and isotopic compositions. Litterbags were placed in aerated, oxic seawater in the dark, as well as buried in both surface (0-5 cm depth) and subsurface (> 10 cm depth) sediments of a tidal creek on Sapelo Island, GA.

Senescent *S. alterniflora* and *J. roemarianus* leaves were collected from a salt marsh near Sapelo Island, GA on two separate occasions, for either the lab incubation or the *in situ* sediment incubation. Leaves were air dried, and finely cut into small pieces (roughly 2 mm x 2mm) prior to the experiments.

The effects of decomposition in seawater were investigated by adding cut pieces of each plant leaf into respective flasks. Replicate flasks were prepared for each sampling point, for a total incubation period of 1 year. The flasks were filled with seawater (30 ppt) that was collected prior to the study at the mouth of the Altamaha River, GA, USA. The incubation was carried out in a temperature-controlled incubator at 16°C and in complete darkness. The flasks were maintained aerobic by continuously purging with air. At each time point, the duplicate samples were centrifuged and the litter pellet was collected for further analyses.

The effects of decomposition in sediments were investigated by in situ incubating litterbags in surface (0 - 5 cm) and subsurface (> 10 cm) sediments for 1 year in a creek of the Sapelo Island. Senescent plant leaves (*S. alterniflora* and *J. roemarianus*) were collected, freeze-dried, finely cut and placed into separate litterbags constructed from 100µm polypropylene mesh. Duplicate litterbags containing *S. alterniflora* and *J. roemarianus* were attached to PVC pipe using zip-ties and incubated on surface sediments. A mesh box was placed around the litterbags to reduce disturbance of litterbags by macrofauna. Replicate litterbags of *S. alterniflora* and *J. roemarianus* were also attached to PVC pipes and incubated at a depth of 10 cm below the sediment surface for subsurface sediment degradation studies. Replicate litterbags incubating on surface sediments, and one PVC pipe with attached buried litterbags were harvested at each sampling period. The litter samples were collected throughout the incubation period and immediately frozen on dry-ice for further analyses.

2.2. Elemental analyses and bulk sample isotope measurements

Freeze-dried and ground plant tissues were analyzed without acidification for total organic carbon, total nitrogen content, total organic carbon stable isotope composition, and total nitrogen stable isotope composition by in-line combustion on an elemental analyzer (Carbo Erba CHN analyzer) coupled to an isotope mass spectrometer. Sediment samples were freeze-dried, ground and acidified with HCl prior to elemental analysis. Total organic carbon (OC), total nitrogen (TN), and $\delta^{13}C$ of total organic carbon were measured using the same elemental analyzer.

2.3. Lignin-phenol

Freeze-dried plant and sediment samples were analyzed for lignin-derived phenols using a modified cupric oxide method (Goñi and Montgomery 2000; Hedges and Ertel 1982). Briefly, freeze-dried plants or sediments were oxidized with CuO in alkaline conditions (2 N NaOH) with $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in stainless steel reactor vessels at a temperature of 150 °C for 3 hours. Reaction products were extracted with ethyl acetate. In order to convert polar O-H groups into non-polar, more thermally stable, groups that can be analyzed readily via gas chromatography, exchangeable hydrogens present on compounds in the extract were converted to trimethylsilyl derivatives using BSTFA + 1% TCMS by heating at 60 °C for 10 min. Trimethylsilyl derivatives were analyzed by gas chromatography using a 30 m x 0.250 mm (i.d.) column (0.25 μm film thickness) column fitted to a flame ionization detector. Quantification of lignin-derived phenols was accomplished by comparing the peak area of recovery standards ethyl vanillin and *trans*-cinnamic acid to each individual lignin phenol, and accounting for differences in the response factors of each compound analyzed.

2.4. Lignin parameters

Lignin parameters (Table 3.1) provide information regarding the extent of degradation and on the various lignin degradation pathways. Eleven monomers are produced during the oxidation of lignin with CuO. These monomers are grouped into 4 families: the *p*-hydroxy phenols, vanillyl phenols, syringyl phenols, and cinnamyl phenols. Lignin content, Λ_6, is calculated the sum of vanillyl and syringyl phenols (mg/100 mg OC).

Although the *p*-hydroxyl phenols may predominately originate from lignin, non lignin sources for these compounds have been identified (Hedges and Parker, 1976). Vanillyl phenols are ubiquitous in all lignins, whereas syringyl phenols are produced primarily in angiosperms. Various diagenetic indicators can be calculated using lignin phenols, providing information about the mechanism and pathway of lignin degradation (Chapter 1).

2.5. Lignin compound-specific isotope analysis

The carbon isotope composition of individual lignin phenols was determined by a gas chromatographic isotope ratio mass spectrometry system (GC-IRMS). Analysis was performed on a GC combustion system coupled to a Delta II isotope ratio mass spectrometer. Accuracy and reproducibility of the instrument was monitored through internal standard monitoring using cinnamic acid and 3,4-dihydroxybenzoic acid as internal standards. The stable isotope composition of the carbon in the BSTFA-TMS derivatizing agent and of external lignin standards were measured off-line and used to calculated the $\delta^{13}C$ value of lignin phenols (Goñi and Eglinton, 1996). A greater amount of variability in $\delta^{13}C$ values was observed in 3,4-dihydroxybenzoic acid (± 2 ‰ - ± 5 ‰)

than in cinnamic acid (± 0.7‰ to ± 2 ‰). The increased variability in 3,4-dihydroxybenzoic acid is a result of the number of carbons that get added (Goñi and Eglinton, 1996) on during the derivatization process, relative to the total number of carbon atoms in the molecule (9:16). The largest ratio of C added during derivatization relative to total molecular C in the lignin phenols of interest is (6:14). The error expected increases proportional to the extent of C added during derivatization (Goñi and Montgomery, 2000). The upper estimate of ± 3 ‰ observed for cinnamic acid is used to define the cutoff for a significant change in compound-specific isotope value variability.

2.6. Rate of OC loss and lignin loss calculations

A multi-G model was used to describe the loss of OC and lignin in plant litter. The multi-G model is simply an expansion of a first-order decay model, which accounts for multiple fractions with differing reactivity (Westrich and Berner, 1984). The changes in OC and lignin, as a function of time, were described using the general equation

$$G_T(t) = G_{01} \exp^{(-k_1 t)} + G_{NR} \quad (1)$$

where G_T is the concentration of total OC or lignin, G_{01} is the initial concentration of the reactive OC or lignin fraction, G_{NR} is the concentration of the unreacted (non-degraded) OC or lignin fraction, and $k_1$is the first-order decay constant of the reactive OC or lignin fraction.

3. Results

3.1. Bulk tissue elemental analysis and mass loss in S. alterniflora *and* J. roemarianus *leaf litter*

The mass contents, ash-free dry weight (AFDW), of two plant materials continuously decreased during incubations but the variations differed between plants and with incubation conditions (Figure 3.1). For example, the extent of mass loss was greater for *S. alterniflora* than for *J. roemarianus* in sediments and the mass loss was greater in surface than in subsurface sediments (Figure 3.1). On the other hand, the extents of mass loss were similar for *S. alterniflora* and *J. roemarianus* in seawater.

Trends in the organic carbon content of the litter differed between plants and environments. In surface sediments, an overall moderate decrease in carbon content was observed for *S. alterniflora* (loss of ~10 %; Figure 3.1b) and *J. roemarianus* (loss of ~5 %; Figure 3.1b). This differed from the pattern in anoxic sediments, where *S. alterniflora* increased overall (7 %) and *J. roemarianus* only slightly decreased (-2 %). The greatest changes of %OC were observed in seawater degradation for both *S. alterniflora* and *J. roemarianus* (roughly -60 %), where carbon-rich compounds were preferentially removed (Figure 3.1). The variations in %OC differ in sediments: the OC content of *J. roemarianus* remains relatively unchanged, while *S. alterniflora* litter initially increases in the proportion of carbon-rich compounds and later the proportion decreases. In seawater, the pattern is similar for both plant types. OC content illustrates how carbon pools change in relation to other pools, but does not provide information regarding the extent of carbon loss during degradation. The remaining % OC expresses the percent of carbon that remains from that which was originally incubated. This number was

calculated by multiplying the OC content by AFDW. This enables a correction for the plant matter loss. The variations in the remaining %OC differed between plant type and environmental conditions (Figure 3.1 c, g). The remaining %OC of both plants decreased similarly in seawater but different in sediments: greater loss for *S. alterniflora* than for *J. roemarianus* and greater in surface than in subsurface sediments. OC was lost at a slower rate in litter decaying in sediments than in seawater (Table 3.1). The rate of OC loss were comparable for *S. alterniflora* and *J. roemarianus* in sediments, but the non-reactive OC fraction in *J. roemarianus* litter was greater in sediments

Because the plant materials used for seawater and sediment incubations were collected at different times, the large difference in the initial C:N ratios of plant materials between experiments were observed (Figure 3.1 d, h). However, the variations in the C:N ratios exhibited similar trends in all incubations: an increase in the first two months and followed by a continuous decrease

3.2. Variations in lignin parameters during decomposition

Lignin content (Λ_6 = Σ vanillyl and syringyl phenols; mg/100 mg OC) expresses the proportion of lignin relative to organic matter. Lignin is refractory and therefore degrades more slowly than other components within the organic carbon pool, therefore Λ_6 usually becomes enriched relative to the OC pool during degradation of organic matter. To depict the changes in lignin content relative to the original lignin content during incubations, this parameter was corrected for loss of organic matter. The corrected lignin contents (or remaining Λ_6) decreased continuously in all incubations (Figure 3.2). The proportion of lignin that remained (i.e. it did not degrade) was greater in sediments

than in seawater. The lignin content (% remaining) in *S. alterniflora* litter decayed more rapidly in sediments than in seawater.

The [PON/P] ratios decreased in *S. alterniflora* incubated in both seawater and sediments and in *J. roemarianus* incubated only in sediments, with the one exception that *J. roemarianus* in seawater (Figure 3.2). The decreased ratios observed in these incubation experiments indicated a non-lignin source of *p*-hydroxy phenols. This prevented the usage of [P/(V+S)] to determine if demethylation of the lignin molecule was occurring. However, the [P/(V+S)] ratio could be calculated for *J. roemarianus* litter incubated in seawater (Figure 3.2 d, i) since the [PON/P] ratio did not decrease. The values of [P/(V+S)] increased in this case, indicating that the demethylation pathway was being utilized during the degradation of *J. roemarianus* in seawater. The increases in the $[(Ad/Al)]_v$ and $[(Ad/Al)]_s$ ratios provided insight into the extent of side-chain oxidation (Figure 3.2): in seawater, both *S. alterniflora* and *J. roemarianus* were exposed to the side-chain oxidation degradation pathway and likewise, *J. roemarianus* in surface sediments.

3.3. Stable carbon isotope composition of bulk tissues and lignin-derived phenols

The bulk tissue carbon isotope composition exhibited different patterns based on plant type and the environment where the plant was degraded (Figure 3.3). The $\delta^{13}C$ values for *S. alterniflora* litter decreased under all environmental conditions, with the greatest variation observed for litter incubated in seawater. The $\delta^{13}C$ values of *J. roemarianus* litter remained relatively unchanged during incubation in sediments, but, unlike the trend observed for *S. alterniflora*, they increased during incubation in seawater.

The trends in $\delta^{13}C$ values of individual lignin-derived phenols varied differently between plant type and environmental conditions (Figure 3.4 and Figure 3.5). The $\delta^{13}C$ values of *S. alterniflora*–derived phenols (except the cinnamyl phenols) varied in a relatively narrow range that is smaller than the error range observed for the internal standard (± 3 ‰) during incubation in surface sediments (Figure 3.4 and Figure 3.5, a-d). Ferulic acid and *p*-coumaric acid, however, experienced significant variation (7‰ and 6 ‰, respectively) during incubation in sediments (Figure 3.5 c, d). The *S. alterniflora* that was incubated in subsurface sediments varied slightly less than that in the oxic sediments in *p*-coumaric acid (5‰) and ferulic acid (4‰) phenols (Figure 3.5 c, d). By contrast, the variation in $\delta^{13}C$ values of lignin in *S. alterniflora* litter that was degraded in seawater was substantially larger, ranging from 5 ‰ (acetovanillone) to 20 ‰ (ferulic acid) (Figure 3.5 c, d). The variations in $\delta^{13}C$ values for lignin in *J. roemarianus* were slightly less in seawater, ranging from 3‰ (vanillin) to 11‰ (ferulic acid) (Figure 3.4 and Figure 3.5, e-h). Despite the minimal variation in $\delta^{13}C$ values of lignin for *S. alterniflora* that was incubated in sediments, *J. roemarianus* that was incubated in sediments experienced moderate variations in the $\delta^{13}C$ value of vanillic acid and syringic acid in surface (6‰ and 4‰, respectively) and subsurface (7‰ and 6‰, respectively) (Figure 3.5 e, f).

4. Discussion

4.1. Decomposition of S. alterniflora *and* J. roemarianus *litter in seawater and sediments*

The total organic carbon (% OC) content of *S. alterniflora* and *J. roemarianus* litter that were incubated in seawater were initially similar (Figure 3.1) and plants followed similar trends in composition during degradation. *S. alterniflora* and *J.*

roemarianus preferentially lost organic carbon-rich compounds, to a similar extent. After a year, only 12 % OC remained. While the decay rate was twice as fast in *J. roemarianus* (Table 3.2), the non-reactive fraction was a similar size in both plants, at approximately 20%. The C:N ratios of *S. alterniflora* was initially lower than that of *J. roemarianus*, but both plants experienced an increase in C:N early on, followed by a decrease, resulting in a similar C:N ratio in 1 year (Figure 3.1 d,h). The initial increase in C:N indicates the preferential degradation of nitrogen-rich components, the following decrease, then, represented a phase of relative nitrogen increase as nitrogen-poor compounds are degraded.

Decreases in C:N result from either the growth of bacteria on plant litter, or the relative increased presence of recalcitrant humic substances. Bacteria C:N ratios are ~ 4 (Fagerbakke et al., 1996). As bacterial biomass increases, the increased contribution of bacterial nitrogen causes C:N to decrease. Alternatively, recalcitrant nitrogen-containing humic substances may accumulate (Fioretto et al., 2005 and references therein). The presence of nitrogen compounds can inhibit lignin degradation (Coûteaux et al., 1995). Despite this, lignin degradation was significant in both, *S. alterniflora* and *J. roemarianus*. Despite the larger initial lignin content in *J. roemarianus,* after 1 year of decomposition, both plants lost lignin to a similar extent and possessed a non-reactive pool of similar size (Table 3.2).

Closer examination of the lignin parameters provided information regarding the degradation pathways of decomposing *S. alterniflora* and *J. roemarianus* in seawater. [(Ad/Al)] ratios, for instance, provide information regarding the extent of side-chain oxidation (Hedges et al., 1988; Opsahl and Benner, 1995). During side-chain oxidation of

the lignin molecule subunits, an excess of benzoic acids are produced (relative to fresh plant material) from the oxidative cleavage of C_α–C_β side chain links. This was observed readily, on a short time scale, for the vanillyl phenols in aerobic environments (Hedges et al., 1988), and on a longer time-scale, in aqueous settings, for syringyl and vanillyl (Opsahl and Benner, 1997). *S. alterniflora* and *J. roemarianus* experienced an increase in $[(Ad/Al)_v]$ when incubated in oxic seawater. A slight increase was observed for the syringyl $[(Ad/Al)_s]$ ratios as well (Figure 3.2). This suggests that both plants experienced side-chain oxidation during decomposition in seawater. In addition, however, the lignin in *J. roemarianus* litter appeared to additionally undergo demethylation in seawater.

The vanillyl and syringyl units released from lignin during oxidation with CuO possess methoxy functional units. Upon lignin biodegradation, demethylation of the lignin subunits can result (Crawford, 1981), producing 3,4 and 4,5 diols. These diols are lost from the analytical window. Demethylation results in a reduction in the methoxylated phenols, vanillyl and syringyl, but does not affect non-methoxylated phenols, such as *p*-hydroxy phenols. The parameter, [P/(S+V)] provides a means of calculating the extent of demethylation (Dittmar and Lara, 2001), with an increase indicating demethylation. However, with the exception of *p*-hydroxyacetophenone, *p*-hydroxy phenols can have non-lignin sources. The absence of a non-lignin source must be verified before variations in [P/(V+S)] can be interpreted. The unique lignin source of the *p*-hydroxy phenols can be determined using [(PON/P)] (Dittmar and Lara, 2001) where a decrease in this parameter indicates a non-lignin source of *p*-hydroxy phenols.

With the exception of the *J. roemarianus* that was incubated in seawater, all of the litter experienced a decrease in [(PON/P)], indicating a non-lignin source. Potential

sources of *p*-hydroxy phenols in the other litter samples include algae, and dissolved amino acids in seawater (Hedges and Parker, 1976). The ratio of [(PON/P)] in *J. roemarianus* litter that was incubated in seawater remained unchanged, and [P/(V+S)] could be used to determine the importance of demethylation for this material in seawater.

During the degradation of *J. roemarianus* in seawater, [P/(V+S)] increased (Figure 3.2 i). This indicated that demethylation, along with side-chain oxidation, was an important process during *J. roemarianus* decomposition in seawater. Brown-rot fungi primarily degrades plant matter through the demethylation pathway, but also oxidizes side-chains to a lesser extent. White-rot fungi, on the other hand, perform extensive side-chain oxidation and aromatic ring cleavage (Filley et al., 2000). Fungi, however, are not the predominant degraders of lignin in aqueous environments, instead degradation is controlled by bacteria (Benner et al., 1986). This would suggest that bacterial degradation leads to similar degradation pathways as observed for fungi.

Differences in the patterns of OC content, OC loss, and patterns in the values of C:N ratios were different for *S. alterniflora* and *J. roemarianus* in surface and subsurface sediments. The OC content of *S. alterniflora* litter increased under both incubation regimes, but only the litter decaying in the surface sediments experienced a subsequent (after 0.4 y) preferential loss in carbon-rich compounds. On the other hand, the relative OC content of *J. roemarianus* remained relatively constant throughout the study period. This does not imply that carbon was not lost, but rather that carbon-rich compounds were lost at a similar rate to relatively carbon-poor compounds, maintaining a steady signal. The extent of OC loss differed for the two plants and *J. roemarianus* seemed to be less labile. *J. roemarianus* litter possessed a larger OC pool during decomposition than did *S.*

alterniflora (Table 3.2), despite similar decay rates. Likewise, lignin decayed faster and to a greater extent in *S. alterniflora* than in *J. roemarianus* and the lignin non-reactive pool in *J. roemarianus* was twice that of *S. alterniflora*, following a similar trend to that observed in OC loss.

The extent of lignin degradation may be linked to the lability of plant matter, as indicated by C:N ratios, and may explain why lignin in *S. alterniflora* degraded to a greater extent. Although *S. alterniflora* litter initially had a greater C:N ratio than *J. roemarianus*, after 0.2 y the C:N ratio of *S. alterniflora* litter decreased in surface and subsurface sediments. However, *J. roemarianus* litter in subsurface sediments maintained a fairly constant C:N ratio, while litter in surface sediments only experienced a minor decrease. One possibility is that C-rich and N-rich compounds are being degraded at a similar rate. Alternatively, a lack in increased nitrogen content could signify low bacterial biomass abundance. As bacteria are the predominant degraders of lignin and plant biomass in sediments and seawater (Benner, Newell, et al., 1984; Newell et al., 1989), low bacterial biomass abundance would result in slower degradation.

Comparing C:N ratios for starting material of *S. alterniflora* incubated in seawater with that incubated in surface and subsurface environments, it is evident that these starting materials were at differing degradation states. The starting material of *S. alterniflora* that was incubated in seawater, exhibited characteristics of material more degraded than the material incubated in sediments. The C:N ratios of *S. alterniflora* decrease with degradation (Haddad et al., 1992), and the C:N ratio of the *S. alterniflora* starting material that was incubated in seawater was lower than that of the sediment material. The seawater and sediment incubations were begun at separate times and the

starting plant material was collected separately. It is possible the leaves collected for the seawater incubation had undergone more degradation.

4.2. Effects of decomposition of S. alterniflora *and* J. roemarianus *on their geochemical characteristics and isotopic signals*

The stable carbon isotope composition of bulk *S. alterniflora* and *J. roemarianus* tissues indicated different degradation process take place for the plants during degradation in seawater The bulk tissue $\delta^{13}C$ values of *S. alterniflora* decreased as a result of two processes (Figure 3.3). On the one hand, the loss of more labile but isotopically lighter material, cellulose, results in a decrease in $\delta^{13}C$ values as the relative lignin fraction increases (Benner et al., 1987). On the other hand, the lignin $\delta^{13}C$ values in *S. alterniflora* became more negative with increasing degradation. In contrast, the $\delta^{13}C$ value of *J. roemarianus* bulk litter increased with degradation in seawater (Figure 3.3). While the $\delta^{13}C$ value of lignin in *J.* roemarianus does increase during degradation (Figure 3.4 and Figure 3.5), this increase is insufficient to account for the change in $\delta^{13}C$ of total organic carbon. Instead, the observed $\delta^{13}C$ values may be explained by the growth of microbial biomass.

The $\delta^{13}C$ values of lignin phenols from *S. alterniflora* that was degraded in seawater consistently decreased, with changes varying from 5-12‰ (Figure 3.4 and Figure 3.5). This differed with the observations of *S. alterniflora* degraded in sediments, where the $\delta^{13}C$ values did not vary to appreciable magnitude. The differences in the trends in carbon isotope compositions of *S. alterniflora* in these environments may result from differences in the lignin degradation pathway employed. On the timescale of the study, the side-chain oxidation was not observed for *S. alterniflora* incubated in

sediments, however lignin loss was observed. This loss could have occurred from solubilization of lignin, which can occur in anaerobic environments (McSweeney et al., 1994) or through aromatic ring cleavage. The lignin peroxidases and manganese peroxidases known to initiate ring cleavage require oxygen (Jeffries, 1994). Aromatic ring cleavage in sediments would require the presence of oxygen or novel enzymes. Evidence of side-chain oxidation and of ring cleavage was evident for *S. alterniflora* degraded in seawater.

During side-chain oxidation, phenyl propanoid units are oxidized to carboxylic acids (Crawford, 1981). During degradation involving side-chain oxidation, the lignin-derived benzoic acid pool increases, and $\delta^{13}C$ values of the benzoic acids are dependent on carbon isotope composition of the precursors. For instance, several pathways lead to the formation of vanillic acid: depolymerization of the lignin molecule, yielding vanillic acid, oxidation of the aldehyde precursor (vanillin), and microbial $C_\alpha C_\beta$ cleavage to monomeric vanillic acid (Godden et al., 1992). Each of these pathways, then, could potentially produce the same molecule, but with differing $\delta^{13}C$ values. The various acid formation pathways may contribute to the average $\delta^{13}C$ signal of vanillic and syringic acids respectively, during the degradation of *S. alterniflora* in seawater, and result in variable $\delta^{13}C$ values over time.

The stable carbon isotope compositions of the ketones and aldehydes from lignin in *S. alterniflora* that was incubated in seawater varied appreciably and also decreased during the incubation period (Figure 3.4). The change in $\delta^{13}C$ values of the lignin phenols during the degradation of lignin may be the result of the aromatic ring cleavage that occurred in conjunction with side-chain oxidation. Degradation of the lignin incubated in

sediments occurred through aromatic ring cleavage or solubilization, but in these systems $\delta^{13}C$ did not change. The isotope fractionation resulting from the degradation of a compound can vary depending on the degradation conditions and on the degradation pathway. Enrichment factors for the biodegradation of benzene differ significantly for different organisms using the same pathway, for different degradation pathways, and for degradation in different redox conditions (Fischer et al., 2008). It is possible, then, that differences in the paths utilized to degrade the lignin in seawater versus surface and subsurface sediments may result in a depletion in $\delta^{13}C$ values of lignin in seawater, but an unappreciable change in sediments. Alternatively, the extent of degradation may control isotopic variation in these different systems. The *S. alterniflora* that was incubated in seawater exhibited characteristic signs of increased degradation relative to the *S. alterniflora* incubated in sediments. The potential for greater change in $\delta^{13}C$ of lignin products in sediments should not be ruled out.

Isotope fractionation during degradation often causes the $\delta^{13}C$ value of unreacted precursor to increase as bonds between lighter isotopes are more easily broken (Meckenstock et al., 1999), and a concomitant decrease in the $\delta^{13}C$ values of products occurs. Vanillic acid and syringic acid, products of side-chain oxidation reactions, exhibited the predicted trend (Figure 3.5) during degradation of lignin in *S. alterniflora* degrading in seawater. As the products of side-chain oxidation accumulate in the vanillic acid and syringic acid pools, their $\delta^{13}C$ value should decrease. Conversely, the ketones and aldehydes present are the unreacted constituents that remain unaltered from side-chain oxidation and ring cleavage. Enrichment in ^{13}C was expected for these molecules,

consistent with the observed depletion (Figure 3.4). Another possible explanation for the depletion in ^{13}C is the heterogeneous distribution of lignin throughout the cell.

Lignin heterogeneity can occur within a given cell wall, where lignin monomer composition and the overall quantity of lignin deposited can vary depending on location (Campbell and Sederoff, 1996 and references therein). Additionally, the predominant monolignol can differ based on cell type, whether fiber cells or tracheary elements (Weng and Chapple, 2010 and references therein). Finally, lignin deposition within plants does not occur uniformly. Instead, lignin is deposited sequentially during the secondary thickening of cell walls. Secondary cell walls consist of 3 layers and lignin deposition occurs in different phases, following the deposition of carbohydrates, and is deposited differentially in two of the three cell wall layers (Boerjan et al., 2011). Variation in the $\delta^{13}C$ of the lignin precursors throughout these steps—whether formation of different cell types or formation of different cell layers—could result in a heterogeneous distribution of ^{13}C throughout the cell wall and between cell types. In addition, the initial degradation of middle cell wall layers would cause variations in the $\delta^{13}C$ value of the remaining lignin. However, our studies do not provide sufficient information to determine if these processes are occurring.

Unlike the consistent decrease in $\delta^{13}C$ values during degradation of *S. alterniflora* in seawater, the $\delta^{13}C$ values of lignin phenols in *J. roemarianus* did not show a clear increasing or decreasing pattern (Figure 3.4b and Figure 3.5b). While *S. alterniflora* was most likely predominately degraded by white-rot fungi, *J. roemarianus* was more likely degraded by brown-rot (see review in Filley et al., 2000), leading to a more complex degradation pattern. Three pathways of lignin degradation were evident for *J.*

roemarianus degrading in seawater: demethylation, side-chain oxidation, and aromatic ring cleavage. The influence of all these pathways complicated the lignin isotope dynamics. As a result, vanillic acid and acetosyringone, the compounds with an appreciable variation in $\delta^{13}C$ values (> 3‰), did not exhibit clear patterns of increase or decrease in $\delta^{13}C$ values. However, a large variation in vanillic acid (5‰) and acetosyringone (7‰) was observed, and the syringic acid increases during the incubation period by 7‰.

In surface sediments, where lignin in *J. roemarianus* underwent side-chain oxidation and aromatic ring cleavage or solubilization, the trends in lignin isotope composition resembled that of seawater degradation. Vanillic acid did not show a clear pattern, but varied overall by 6‰, and syringic acid increased by 4 ‰—other lignin phenols did not vary to an appreciable amount. The similarity in decomposition trends, between *J. roemarianus* in surface sediments and seawater, despite having been degraded in different environments, and by potentially different organisms, suggests that the degradation pathways were similar and the distribution of lignin in *J. roemarianus* may limit the extent of ^{13}C fractionation during degradation.

5. Conclusions

Differences in lignin phenol composition and $\delta^{13}C$ values for *S. alterniflora* and *J. roemarianus* in seawater, surface sediments, and subsurface sediments can be summarized as follows:

1. Rates of lignin degradation and OC loss differed based on plant type and environment. First-order decay rates for the reactive fraction were generally

greater for *S. alterniflora* than *J. roemarianus* and generally greater in seawater.

2. Degradation pathways of lignin differed based on plant and type of environment. Side-chain oxidation and aromatic ring cleavage were the dominant pathways for *S. alterniflora* in seawater, while *J. roemarianus* lignin was degradaed by demethylation in addition to side-chain oxidation. In surface and subsurface sediments, *S. alterniflora* degraded primarily by aromatic ring cleavage, while *J. roemarianus* degraded by side-chain oxidation and aromatic ring cleavage.
3. Aromatic ring cleavage degradation occurred for *S. alterniflora* in seawater and sediments, but resulting trends in $\delta^{13}C$ values of lignin phenols differed, which could be due to different enzymes degradation pathways used in the different environments.
4. The decrease in $\delta^{13}C$ values of lignin phenols of *S. alterniflora* degraded in seawater may be due to the structural and compositional variability of lignin throughout cells and plant tissue, leading to preferential degradation of lignin that is more enriched in ^{13}C.
5. Patterns in the $\delta^{13}C$ values of lignin phenols from *J. roemarianus* that was incubated in seawater were complicated by the complexity of numerous pathways degrading lignin.
6. Similarities between the change in $\delta^{13}C$ values of *J. roemarianus* lignin in seawater and sediments, where the microbial community are likely to differ,

suggests that the carbon isotope dynamics may have resulted from the plant-specific distribution of lignin.

An unchanging isotope composition of undegraded organic matter is required when using the isotope composition of biomarkers to deduce the source of material in a complex organic mixture. During degradation of lignin, large variability was observed, while the pattern (increase or decrease in $\delta^{13}C$ values) and the affected lignin phenols, differed for plants and for degradation regime. The variability observed between plants and environments make it difficult to predict the expected change in $\delta^{13}C$ value of lignin during degradation. As a work-around, the lignin phenols with the least variability resulting from degradation should be identified and used.

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Table 3.1. Definitions of parameters used.

Parameter	Description	Reference
PON/P	Molar ratio of *p*-hydroxyacetophenone to total *p*-hydroxy phenols	(Dittmar, T., and Lara, R. J., 2001)
P/(V+S)	Molar ratio of p-hydroxyacetophenone to total vanillyl and syringyl phenol	(Dittmar, T., and Lara, R. J., 2001)
$(Ad/Al)_V$	Molar ratio of vanillic acid to vanillin	(Hedges, J., 1988)
$(Ad/Al)_S$	Molar ratio of syringic acid to syringaldehyde	(Hedges, J., 1988)
Λ_6	Sum of vanillyl and syringyl lignin phenols (mg/100 mg OC)	(Opsahl, S., and Benner, R., 1995)
% Initial	Percent initial yields. Values over 100% indicate a relative enrichment	
% Remaining	Percent of of the intial remaining, adjusted for mass lost (AFDW)	

Table 3.2. Experimentally derived decay parameters and carbon isotopic shifts.

	G_{01}	G_{NR}	k_1 (yr^{-1})	$\Delta \delta^{13}C$ (‰)
% OC (remaining)[a]				
S. alterniflora				
surface sediments	0.91	0.098	0.037	2.4[e]
subsurface sediments	0.78	0.20	0.032	2.6[e]
seawater	0.68	0.20	0.057	6.3[e]
J. roemarianus				
surface sediments	0.68	0.31	0.020	0.7[e]
subsurface sediments	0.38	0.59	0.027	0.4[e]
seawater	0.76	0.22	0.12	2.1[e]
Λ_6 (remaining)[b]				
S. alterniflora				
surface sediments	7.8	1.3	3.0	-
subsurface sediments	5.5	2.8	4.5	-
seawater	4.7	0.089	1.9	-
J. roemarianus				
surface sediments[c]	-	-	-	-
subsurface sediments	-	12	0.58	-
seawater	1.3	6.5	2.0	-

a. G_{01} and G_{NR} are presented as fractions

b. G_{01} and G_{NR}, are presented in units of mg/100 mg OC

c. Surface sediments could not be fit well for Λ_6 (remaining)

d. k_1 is the first order decay constant for degrading organic matter

e. $\Delta \delta^{13}C$ is the absolute variation in δ-values during the incubation period

Figure 3.1 Variations in ash-free dry weight AFDW (a), % initial organic carbon (b), % remaining organic carbon (c), and C:N ratios (d) for *S. alterniflora* and *J. roemarianus* litter incubated in surface (gray) and subsurface (black) sediments, and aerated seawater (white).

Figure 3.2 Percent of lignin content (Λ_6; sum of vanillyl and syringyl phenols, mg/100 mg OC) remaining – a, (Ad/Al) ratios for vanillin – b, (Ad/Al) ratios for syringaldehyde – c, P/PON – d, P/(S+V) – e, for *S. alterniflora* and *J. roemarmianus* incubated in surface (gray) and subsurface (black) sediments, and seawater (white) for 1 year. See Table 3.1 for description of parameter notation.

Figure 3.3 Variations in the $\delta^{13}C$ values of total organic carbon for *S. alterniflora* and *J. roemarianus* litter incubated in surface (gray) and subsurface (black) sediments, and aerated seawater (white).

Figure 3.4 Variations in the $\delta^{13}C$ values of lignin-derived aldehyde and ketone phenols from (a) – *S. alterniflora,* and (b)– *J. roemarianus* litter that was incubated in surface (gray) and subsurface (black) sediments, and aerated seawater (white) for 1 year. VAL – vanillin, AVO – acetovanillone, SAL – syringaldehyde, ASO – acetosyringone.

Figure 3.5 Variations in the $\delta^{13}C$ values of lignin-derived acidic phenols from (a-d) – *S. alterniflora,* and (e-h)– *J. roemarianus* litter that was incubated in surface (gray) and subsurface (black) sediments, and aerated seawater (white) for 1 year. VAD – vanillic acid, SAD – syringic acid, p-CAD – *p*-coumaric acid, FAD – ferulic acid.

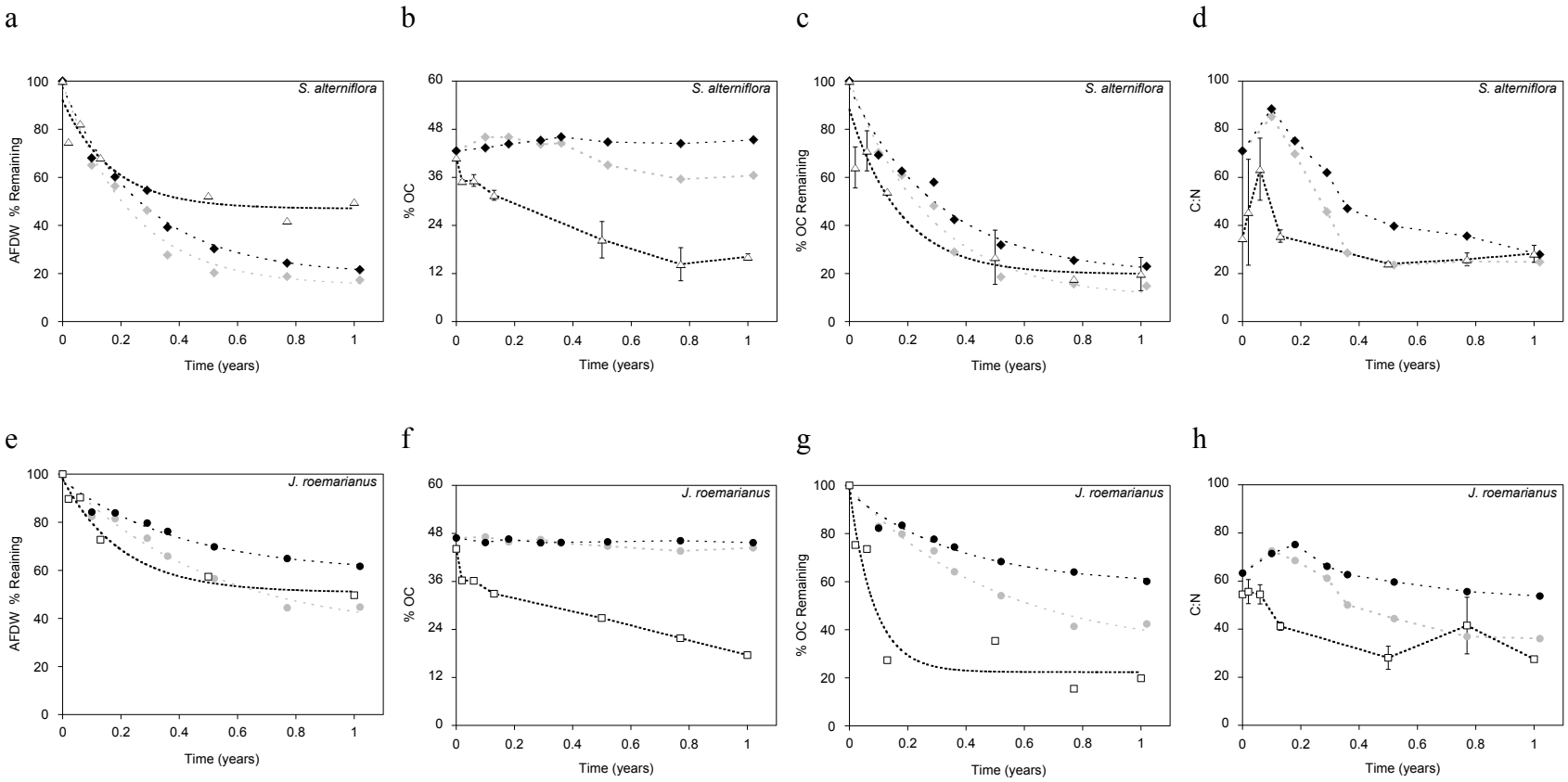
a
b
c
d
e
f
g
h
S. alterniflora
J. roemarianus
AFDW % Remaining
AFDW % Reaining
% OC
% OC Remaining
C:N
Time (years)

Figure 3.1

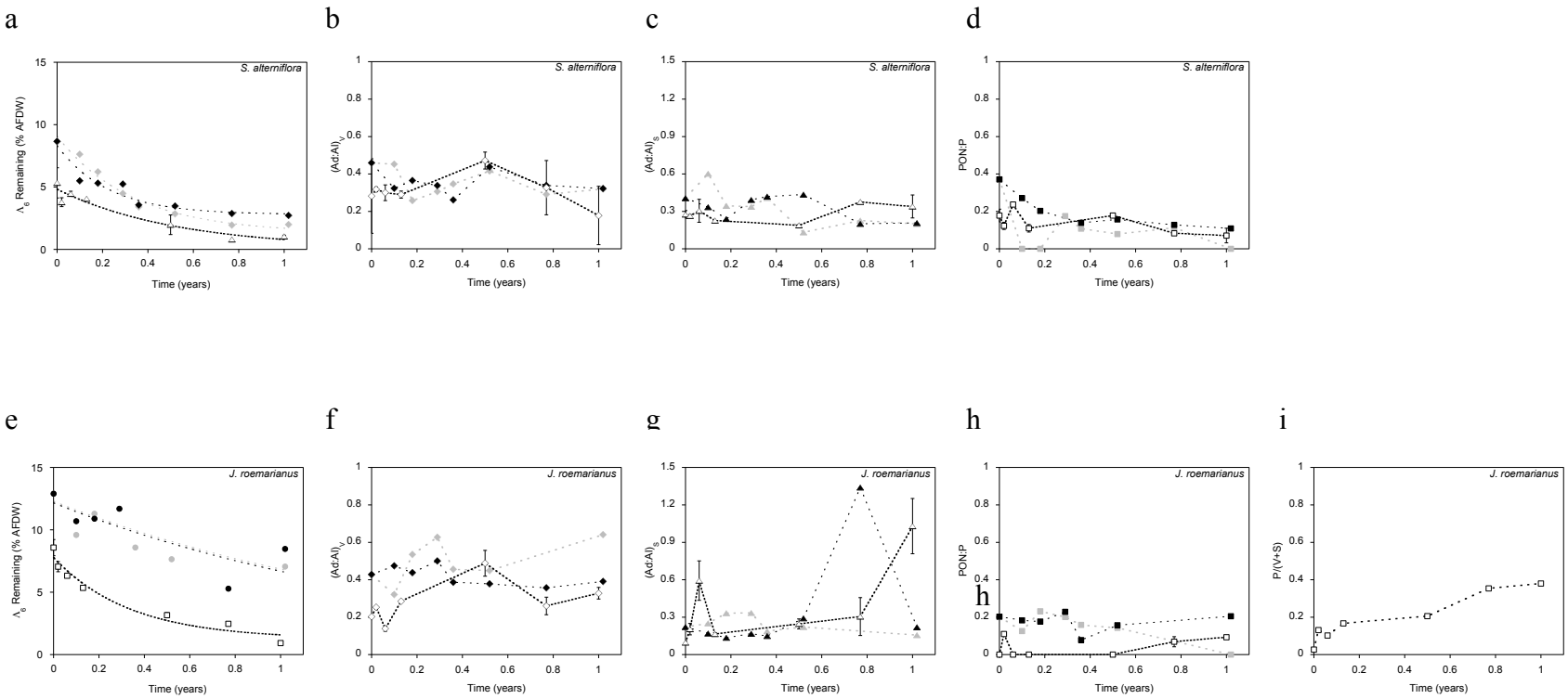
a
b
c
d
e
f
g
h
i
S. alterniflora
J. roemarianus
Time (years)
Λe Remaining (% AFDW)
(Ad:Al)V
(Ad:Al)S
PON:P
P/(V+S)

Figure 3.2

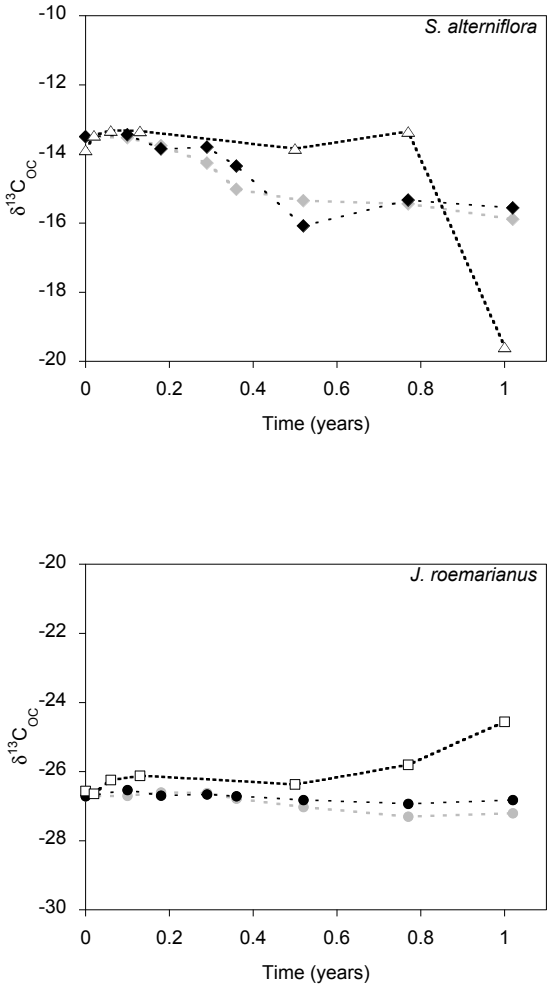
S. alterniflora
-10
-12
-14
-16
-18
-20
δ13COC
0
0.2
0.4
0.6
0.8
1
Time (years)
J. roemarianus
-20
-22
-24
-26
-28
-30
δ13COC
0
0.2
0.4
0.6
0.8
1
Time (years)

Figure 3.3

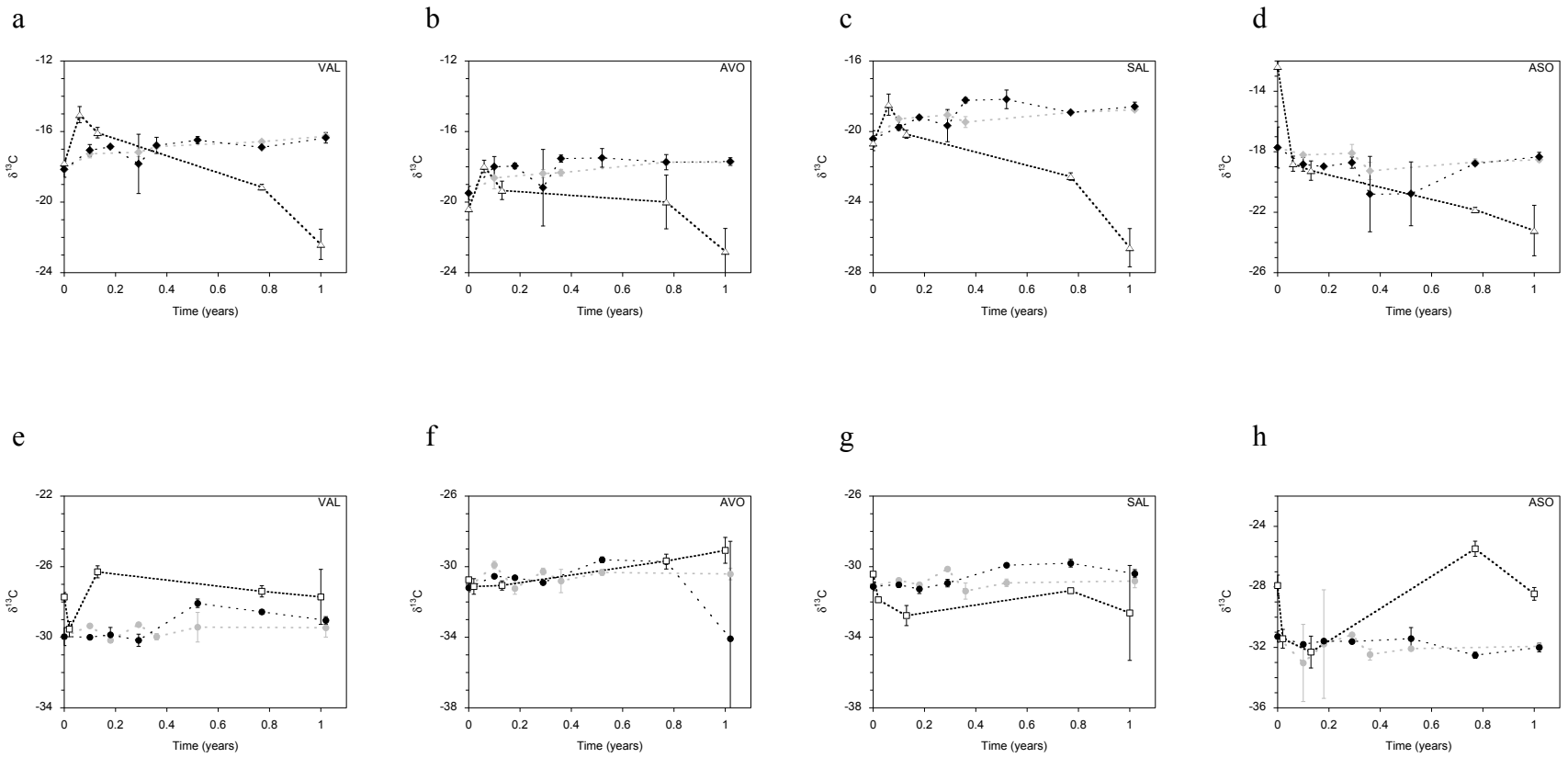

Figure 3.4

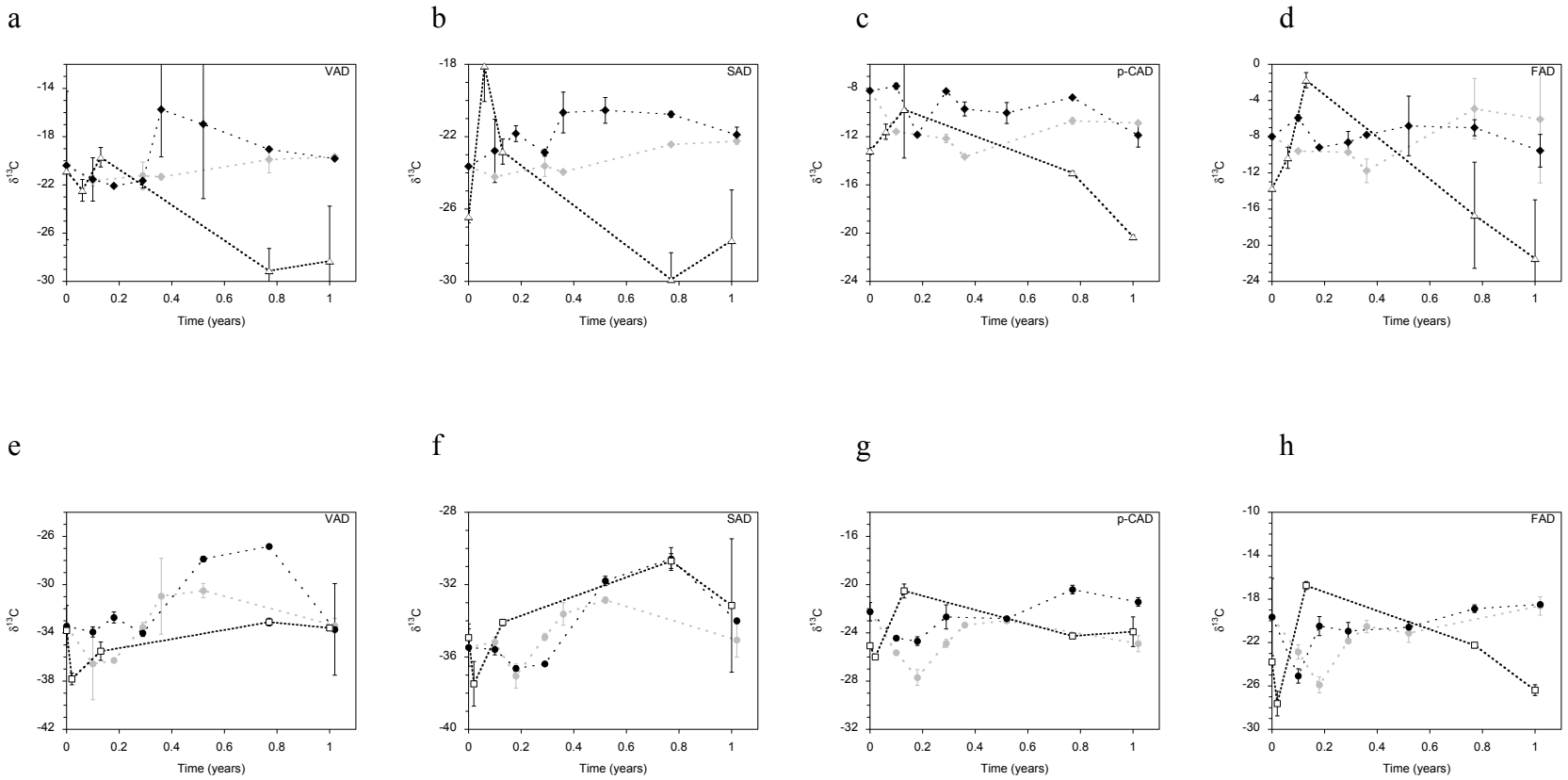

Figure 3.5

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CHAPTER 4

THE USE OF ISOTOPES AND ELEMENTAL RATIOS IN MULTI-PARAMETER MIXING MODELS[3]

[3] Loftis, K. M., C. Meile. To be submitted to Limnology and Oceanography: Methods

Abstract

Stable isotope signatures and elemental ratios are used extensively to describe and identify samples throughout various fields. These parameters are often incorporated into mixing models, which upon solving provide information on the contribution from end-members. The validity of results from multi-parameter mixing models, however, is dependent on the parameters utilized. We show that multi-parameter mixing models require the use of parameters that ensure mass conservation, which precludes direct use of elemental ratios and isotopic signatures.

When the number of parameter exceeds the number of end-members, the mixing model system becomes over-determined and is solved as a least-square problem. As the values of different parameters occur at different scales, the solution procedure may benefit from normalization. We explored the effects of normalization on 21 different mixing models, each composed of different combinations of parameters. In a reanalysis of literature data on organic matter cycling in Dai and Sun (2007), we investigated the impact of parameter choices and treatment on the interpretation of the observational data.

1. Introduction

Stable isotope signatures, chemical concentrations and elemental ratios are parameters that are used extensively in various fields including ecology, geochemistry, hydrology and archaeology to describe the composition of a sample and to deduce the fractional contribution of end-members to a mixture (Boschker et al., 1999; Phillips, 2001; Bunn and Davies, 2003; Goñi et al., 2003; Gordon and Goñi, 2003; Drucker and Bocherens, 2004; e.g. Benner, 2005),. A mixture that is comprised of various sources will

have a signature, for each measured parameter, that reflects the weighted average of signatures of all contributing sources. A system of linear equations can be used to describe the mixture, with each equation defining a measured parameter as a function of the end-member signals and the end-member fractional contribution to the mixture. The determination of the (unknown) fractional contribution from *i* end-members requires *i* equations for a unique solution. A general mass-balance equation that reflects the weighted average of the mixture (X_M) is defined as follows:

$$X_M = \sum f_i X_i \quad (1)$$

where f_i represents the fractional proportion of *i* components in the mixture, and X_i represents the signature of the i^{th} component.

If the number of system descriptors (parameters) exceeds the number of end-members contributing to the mixture, optimal solutions are determined by minimizing the L2 norm. If, on the other hand, there are more end-members than parameters that describe each end-member and the mixture, the system is under-determined system. Phillips and Gregg (2003) described an iterative procedure for calculating the relative contribution of end-members in an under-determined system, where the number of end-members exceeds the number of parameters. Moore and Semmens (2008) also developed a mixing model, based on Bayesian statistical techniques, for dealing with the problem of too many sources and uncertainty in isotope signatures. This method determined the probability distribution for the proportional framework.

In this communication we present an alternative analysis of mixing models, with an emphasis on situations that involve multiple end-members and parameters. We show that this precludes the direct use of elemental ratios or isotopic ratios as model

parameters. Furthermore, we assess the role of data normalization when fitting data, and reassess the fractional contribution of end-members to sediments along the Altamaha River, USA, to illustrate the impact of the above factors on the outcome of mixing model calculations.

2. Procedures

The relative contribution of various sources to the signatures of various parameters can be expressed as:

$$\begin{aligned} X^{m}_{mix} &= g_1 X^{m}_{1} + g_2 X^{m}_{2} + \cdots + g_i X^{m}_{i} \\ &\cdots \\ X^{n}_{mix} &= g_1 X^{n}_{1} + g_2 X^{n}_{2} + \cdots + g_i X^{n}_{i} \end{aligned} \tag{2}$$

where X^{n}_{mix} denotes the composition of the n^{th} parameter in the mixture, X_i reflects the composition of the i^{th} source, and g_i is the fractional contribution of that end-member. If all end-members contributing to the mixture are accounted for, Equation 2 can be supplemented by a completeness constraint,

$$\Sigma\, g_i = 1 . \tag{3}$$

At least i-1 parameters (when Equation 3 holds true) are required to quantify the relative contributions from i end-members. In this way, the system is determined and g_i are solved for explicitly. Note that the fractional contributions, g, refer to the mass contributions of end-members to a mixture and are not parameter dependent.

The fractional contribution of sources, g, is related to the fractional contribution of parameters, f, by

$$f_i^{n} = \frac{X_i^{n}}{X_{mix}^{n}} g_i \tag{4}$$

where f_i^n is the fractional contribution of the n^{th} parameter from end-member i to the mixture, X_i^n is the mass fraction of parameter n in the i^{th} end-member or the mixture, respectively. More explicitly, f is defined as the proportion of parameter n from end-member i in the mixture, whereas g is defined as the proportion of end-member i in the mixture. Therefore, g_i is the same for any chemical used in the mixing model, which is not the case for f_i. For example, if a mixture containing A and B is made up, in part, of an end-member that contains element A but not B, the end-member contributes to the mixture for pool A but not for pool B. For this reason, when fractional contributions are parameter dependent, f_i differs for the governing equations for parameters A and B, thus leading to more unknowns than equations (an underdetermined system). It is, therefore, necessary to use fractional contributions that represent the end-member (not the parameter) when multiple elements are used in the system equations. The necessity to use fractional source contributions, g_i, in conjunction with multiple parameters has implications for the use of isotopic signatures as well as elemental ratios.

In any mixture, parameter ratios (e.g. carbon-to-nitrogen, C:N) can be computed as

$$\frac{A_{mix}}{B_{mix}} = \frac{g_1 A_1 + g_2 A_2 + \cdots + g_i A_i}{g_1 B_1 + g_2 B_2 + \cdots + g_i B_i} \tag{5}$$

where A and B represent parameters. This, in general, differs from

$g_1 \frac{A_1}{B_1} + g_2 \frac{A_2}{B_2} + \cdots + g_i \frac{A_i}{B_i}$, showing that elemental ratios are not to be used in a straightforward fashion in multi-parameter mixing models. Notably, the fractional contribution of end-members is accurately identified in terms of parameters, i.e.

$$\left(\frac{\mathrm{A}}{\mathrm{B}}\right)_{\mathrm{mix}} = f_1\left(\frac{\mathrm{A}}{\mathrm{B}}\right)_1 + f_2\left(\frac{\mathrm{A}}{\mathrm{B}}\right)_2 + \cdots + f_{\mathrm{i}}\left(\frac{\mathrm{A}}{\mathrm{B}}\right)_{\mathrm{i}} \quad (6)$$

but f, the proportion of a parameter from end-member in the mixture, are not the same for different parameters.

3. Assessment

3.1. Use of elemental ratios and δ values

To illustrate the parameter dependence of the fractional contribution variable when an elemental ratio, and thus Equation 6, is used, three mixtures were created by combining two end-members in equal parts (g_1=g_2=0.5), alternating the end-members used for each of the three mixtures. Parameter signatures measured on *Avicennia germinans* (mangrove leaves) (Loneragan et al., 1997; Dittmar and Lara, 2001), *Spartina alterniflora* (cordgrass) (Hopkinson and Schubauer, 1984; Currin et al., 1995; Middelburg et al., 1997), and Gulf of Mexico near-shore sediment (Gearing et al., 1977; Bianchi et al., 2007), were used to represent a range of organic carbon and nitrogen isotope compositions, and values of organic carbon (OC) and total nitrogen (N) content readily measured in environmental settings. The carbon and nitrogen contents of each mixture were calculated, separately, using Equation 1 for each of the parameters. The C:N ratio of the mixture was then calculated for each of the mixtures. Using the C:N ratio of the mixture and of the end-members, and assuming Σf_{i}=1, Equation 6 was used to calculate f_1 and f_2 (Table 4.1). In comparing the values of f with those of the known, imposed values of g, it is clear that when ratios are used in a linear mixing model, the proportional contribution that is calculated is that of parameter, not of end-members. Thus, the use of C:N ratios or δ values, whose definition also contains a ratio, are

reserved for a one-parameter system, and are then restricted to the use of f. Therefore, these ratios may not be used when solving for end-member fractional contribution to the mixture, g_i, nor can they be used in a multi-parameter mixing model.

3.2. Fractional contributions of parameters (f) or of sources (g)

To illustrate the impact of using fractional contribution of parameters (f_i), in multi-component mixing models, instead of the fractional contribution of sources (g_i), mixtures of equal portions of two end-members were designed using the above-mentioned characteristics of mangroves, cordgrass and sediment. The fractional contributions (h_i, which represent f_i or g_i) were then calculated using the following combination of parameters, listed with the key findings (see Figure 5.1 and below for detailed results):

(i) Isotopic signatures: employing $\delta^{13}C$ or $\delta^{15}N$ and the completeness constraint, $\Sigma f_i=1$, gives a correct value for f_i (Figure 4.1, upright triangles). However, because $\delta^{13}C$ and $\delta^{15}N$ do not represent true concentrations, the use of g leads to erroneous results. Instead, using ^{13}C or ^{15}N concentrations gives the correct value for g_i (Figure 4.1, circles).

(ii) Elemental content: Used with $\Sigma g_i=1$, gives the correct values for g_i, but not f_i.

(iii) Isotopic signature, elemental content and completeness: Using $\delta^{13}C$ (or $\delta^{15}N$) estimates the values of f (Figure 4.1, upside-down triangles), while using ^{13}C (or ^{15}N) concentrations gives correct values for g (Figure 4.1, crosses).

Values for the parameters identified in each scenario were plugged in for end-members and the mixture, and upon rearrangement, fractional contributions were calculated. In determined systems (i, ii and iv), f_i and g_i were solved for algebraically, while the over-determined system (iii) was solved in the least-square sense using the MATLAB function lsqlin (MathWorks, Inc., 2009)

When isotope content (^{13}C or ^{15}N), elemental content (OC or N), with/without the completeness constraint $\Sigma g_i=1$ were used in a mixing model, the same correct value of 0.5 was obtained in all scenarios for the parameter-independent end-member contribution g_i. However, when using $\delta^{13}C$ or $\delta^{15}N$, the proportional contribution does not produce values of 0.5 (triangles) because their use in Equation 2 does not reflect mass conservation but rather the contribution of an end-member's signal to the signal of the respective parameter in the mixture (Figure 4.1).

Estimates of f_i are dependent on the parameter utilized in the mixing equation, and in the case when δ values are utilized, on the elemental concentration in the end-members. For instance, when the carbon concentration of the two end-members is similar, f_i (Figure 4.1, upright triangles) closely approximate g_i (Figure 4.1, mangrove and cordgrass). However, as the carbon concentrations of the two end-members diverge from one another, f_i values no longer approximate the end-member fractional contribution (Figure 4.1, sediment and mangrove leaves or cordgrass). In this case, it becomes evident that f_i estimates the parameter fractional contribution, as opposed to the mass fractions. When isotope values (δ values) are used in conjunction with elemental composition, the values are similar to f_i, but not equal (Figure 4.1, upside-down triangles). For example, when mangrove leaf tissue (high in OC content) is mixed with sediment (low in OC

content) in equal mass proportions, the value of f_1 is close to 1 (Figure 4.1). Because mangrove leaves have a high organic carbon content, the leaf tissue will contribute substantially more to the carbon isotope signal of the mixture than the sediment. Thus, the $\delta^{13}C$ value of the mixture will more closely resemble that of mangrove leaves.

The setback in using multi-parameter systems when solving for *f* becomes also evident upon comparing the values of *f* calculated using carbon isotope values ($\delta^{13}C$; Figure 4.1 left panel) and values of *f* calculated using nitrogen isotope values ($\delta^{15}N$, Figure 4.1 right panel). Here, the calculated *f* values differ substantially from each other and from the correct solution. Thus, the *f* solved for in different equations of a mixing model system (such as Equation 2), are different for each parameters thereby leading to more unknowns than equations.

Phillips and Koch (2002) recognized the inconsistency in f_i estimates, arising from variations in the concentration of elements within the end-members and presented a concentration-weighted model:

$$f_{i,x} = \frac{f_{i,\text{Phillips}}[X]_i}{f_{i,\text{Phillips}}[X]_i + f_{j,\text{Phillips}}[X]_i + \cdots + f_{i,\text{Phillips}}[X]_n}$$

where i, j…n denote each end-member contributing to a mixture. Through this transformation, $f_{i,\text{Phillips}}$ are close approximations of the correct mass fractional contributions, g_i. (see Appendix A)

3.3. Normalization

The least squares method calculates the solution to an over-determined system by minimizing the sum of the squares of the error made for all equations. The parameters

used in the least squares regression, however, vary in their magnitude; thus it may be necessary to normalize the parameters so that all of them contribute similarly.

A hypothetical organic matter mixture was constructed by mixing equal parts of three environmentally relevant end-members, representative of characteristic C_3-type plants, C_4-type plants, and phytoplankton. Using data from Loftis and Sun (unpublished) for plant end-member parameters, Redfield stoichiometry to estimate the OC and TN content of phytoplankton, and data from Lehmann et al. (2002), each end-member was characterized by a ^{13}C and ^{15}N, total OC, TN and lignin content. Mixtures consisting of 1/3 C_3, 1/3 C_4, and 1/3 phytoplankton were then constructed using 21 different combinations of parameters, with a varying number of parameters utilized to describe the system (Table 4.1).

To examine the consequence of variability in the measured mixture values (e.g. due to heterogeneity), Monte Carlo simulations (10^5 realizations) were implemented in which the signature of the mixture was varied, chosen randomly from a distribution that was centered at the mean and distributed amongst one standard deviation. The error ascribed to each of the mixture parameter values was defined as 10% of mean parameter values commonly measured in environmental sediments. Next, optimal source fractions were computed based on the known end-member values, $\Sigma g_i=1$, and the additional constraint that $0 \leq g_i \leq 1$.

Two data normalization schemes were investigated for the effect of scaling data to the same magnitude on estimates of g_i, and were compared to results from a non-normalized data set. In the first, the data was scaled by the mean value of each parameter in the mixture, which does not require any additional data than what is already required

for the mixing model. The second method shifted the data by the mean of each parameter in the mixture and scaled by the uncertainty in the mixture (z-score). The results from the Monte Carlo simulations all center on the one-third contribution from each end-member (Figure 4.2) The range in estimates, however, varied between models and normalizations. Both normalizations reduced the spread, with the z-score normalization producing results least sensitive to uncertainties in the mixture (Figure 4.2), last box for each model).

3.4. Optimal choice of source parameters

Despite the observed reduction in the spread of estimated values of g_i, a few models produced consistently large variability, independent of how the data was treated. Quantification of source contributions requires that the end-members are clearly distinguishable. The difference between end-member parameter values can be quantified by how far apart the vertices formed by the end-member values are located from each other. As discussed above, because of the difference in the magnitude of different parameters, they were standardized to so that the largest value of any end-member equaled 1. For both the 2-parameter models (1-10; Table 4.2) and the 3-parameter models (11-16), the three end-members represent a triangle in a 2- and 3-dimensional space, respectively. In order to compare 2-parameter and 3-parameter models, the triangle areas were standardized to the maximum possible areas. In 2-dimensional space, the maximum possible area is defined by a triangle, and in 3-dimensinoal space, the maximum possible area is defined by the face of a tetrahedron (0.5 for 2D, sqrt(3/4) for 3D). We further propose for comparison between models with different numbers of end-members to

compare the realized volume to the maximum possible volume, 1/n!, where n is the dimension (number of parameters).

The correlation between area and spread from Monte Carlo simulations was determined for the three end-members. Area was negatively correlated with the spread in estimated g_i values (Table 4.3), indicating that the models exhibiting the largest spread in estimates of end-member contribution were those that had the most similar parameter signals. This is evident when comparing, as an example, models 1 and 2. Model 1 has a small spread in Monte Carlo simulation results (Figure 4.2) and the area defined by the standardized end-member parameter values (Figure 4.3) is large. On the other hand, model 2 has a large spread (Figure 4.2) and the area defined by end-member parameter values is small (Figure 4.3). Figure 4.4 depicts the relationship between the area bound by end-member parameter values and the spread in Monte Carlo simulation results for the three end-members. The model results became more constrained (less error) as the defined area increased. When the standardized (largest parameter value equals 1) and normalized area bound by parameter values was greater than 0.1, the spread in estimated values plateaued to a standard deviation of less than 10% of the mean value.

4. A reassessment example

The datasets of Dai and Sun(Dai, 2005; Dai and Sun, 2007) were reanalyzed and the proportion of end-members to sediments along a transect, from the Altamaha River (site A) to a site off the river mouth (site I), was recalculated for two study periods. Dai and Sun (Dai and Sun, 2007) estimated the relative proportion of three organic material end-members (*Skeletonema costatum*, marine plankton (MP); *Festuca arundinacea*, land plant-C_3 (LP); and *Spartina alterniflora*, salt marsh plant-C_4 (SMP)) to several sites along

the Altamaha River in the Southeastern United States using $\delta^{13}C$ and C:N ratios. The system was constrained such that the entire organic matter pool in the sediments was composed of the three end-members, $\Sigma f_i = 1$ (i = marine diatom, land plant, or salt marsh plant).

In this reassessment, the data was transformed to ^{13}C content, OC content, and TN content (Gosselink and Kirby, 1974; Wieder et al., 1983). This data can be presented in two ways; the parameters can be expressed in terms of gram sediment, or the parameters can be expressed in terms of gram OM. Lacking knowledge of the organic matter content of the sediment and end-members, the parameters in this model were expressed in terms of total sediment. As sediment is composed of both inorganic and organic matter the end-members contribution sum up to less than 100% of the sediment, and $\Sigma g_i = 1$ is no longer applicable. Thus, a 3 end-member, 3 parameter system was solved to calculate g_i and the completeness constraint (Equation 3) was not utilized. Instead, $\Sigma g_i \leq 1$, which was implemented through the inequality constraints in lsqlin (MathWorks, Inc., 2009). Once g_i were calculated in terms of total sediment, they were converted to express the relation of the three end-members to one another which permitted comparison to the f_i Dai and Sun (Dai and Sun, 2007) calculated.

The estimated fractional contribution of the three end-members differed somewhat from published estimates (Figure 4.5). The previously calculated fractional contributions were calculated not as the fractional contribution of the end-members, but rather as the fractional contribution of parameters to the distinct parameter pools. As demonstrated above, fractional parameter contributions are specific to each, individual parameter, causing the observed differences between g and f. Minor differences were

observed between the two methods for the March dataset. The greatest differences were observed at sites A, D, and G, where the relative proportion of salt marsh plant matter increased, while, inversely, the marine plankton component decreased. These findings are consistent with the lipid distributions for these sites (Dai and Sun, 2007), where terrestrial lipids were greater than those of algal lipids. The greatest observed difference was seen for the October data set, across all stations. The general trend was a relative increase in the marine plankton component, with the exception of sites A, D, and H, where marine plankton decreased.

The marine plankton contribution at station D in October was absent when g_i were calculated despite an approximate marine plankton contribution of 40% when f_i were calculated. In the original calculations, *f*, a spike in the salt marsh plant component, relative to that of neighboring sites, was observed, but not to the extent we observed. Because *f* and *g* are related (Equation 4), it would not make sense to have a value of 0 for *g* and a value >0 for *f*. It is impossible for an absent end-member to contribute to the parameter signal. In our calculations the content of each parameter (^{13}C, C, and N), in the sediment or end-member, was used to calculate *g* explicitly (determined system), providing an accurate view of the end-member distribution in the sediment. The observed difference between the original results at D and our recalculation may be an artifact of how *f* were solved for. In their mixing model, Dai and Sun (Dai and Sun, 2007) using a multi-component, multi-parameter system when solving for *f*.

The end-member distribution at station H in October differed substantially from previous estimates. In our reassessment, the marine plankton component decreased precipitously, and salt marsh plant and land plant contributions increased. This site is

located at the mouth of the Altamaha River, where the marine component of the sediments would be expected to be greater. Although our estimates of the proportion of end-members contributing to station H differed from the previous mixing model estimates, our estimates corroborate with the PCA analysis (Dai and Sun, 2007). Principal component analysis revealed that the organic matter collected at this was predominately degraded material. A greater terrestrial component is consistent with more degraded organic matter, since algal biomass is more labile and would be preferentially lost. At station I, this trend was reversed, and marine plankton contribution predominated that of the other two end-members. This is consistent with station I being located off the river mouth.

5. Summary

The parameter-dependent fraction f_i can be used in a single-parameter system to describe the contribution of individual end-members to the specified parameter signal in a mixture, but are not applicable in multi-parameter mixing models. There, mass fractional contribution, g_i, need to be calculated, as they are applicable for mixing end-member containing different parameters. As a consequence of this distinction between 'fraction of parameter signatures' and 'fractional mass contributions', isotopic signatures and other measures that contain ratios are only applicable for one-parameter mixing models (i.e. 2 end-member models), or else need to be decomposed into individual isotope or elemental balances.

Mixing-model approaches rely on solving for end-member fractional contributions. When using more parameters than end-members, i.e. when dealing with

over-determined systems, normalization of the data is important and improves the determination of mixing fractions under uncertainty.

The optimal choice of parameters to be used in mixing models depends on the ability to accurately distinguish end-members. Using the span between them provides a direct measure thereof. Typically, addition of more parameters tightens the constraints on the system. At the same time, least-square solutions can also suffer from fitting data with little additional information. Our proposed measure presents a means for calculating this spread in a manner that can be compared along varying dimensions. This provides a method for a priori investigating, and selectively choosing, parameters to be used in a mixing model.

Correctly identifying and defining the proportionality parameter being calculated is pertinent in the use of end-member mixing models. Reassessment of models using the end-member fractional contribution parameter, g_i, may be necessary in cases where parameter fractional contribution parameters were used in multi-parameter mixing models. In these cases, the proportionality values were improperly defined, at best. However, for systems where multi-component, multi-isotope mixing models were constructed, and f (instead of g) were solved for, the results lack valid physical interpretation and do not reflect either proportionality variable.

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Table 4.1. Three mixtures were constructed, each composed of 2 end-members. The relative contribution of each end-member to the mixture, g, was 0.5. The C:N ratio of each mixture was calculated by calculating the C content and N content of the mixture, based on g_1 and g_2, and the C and N content of both end-members. Equation 6, was used to calculate f_1 and f_2, with the constraint, $\Sigma f = 1$. Here, f_1 and f_2 are the relative contributions of the C:N signals from end-member 1 and 2, respectively, to the C:N signal of the mixture.

End-member 1	**End-member 2**	$g_{1,true}$	$g_{2,true}$	**C:N of end-member 1**	**C:N of end-member 2**	**C:N of mixture**	$f_{1\ C:N}$	$f_{2\ C:N}$
mangrove leaves	cordgrass	0.50	0.50	75	39	53	0.38	0.62
mangrove leaves	sediment	0.50	0.50	75	14	66	0.85	0.15
cordgrass	sediment	0.50	0.50	39	14	37	0.90	0.10

Table 4.2. Models used in the analysis of parameter selection and normalization method effects on mixing models. 21 models were constructed using different combinations of parameters to describe the system; the model ID is indicated in the first column. For each model, parameters used to describe the system are denoted with an x. The generalized form of the mass-balance equation for each parameter is: $X_{mix} = \Sigma g_i * X_i$, where X denotes one of the various parameters utilized in the model, X_{mix} is the content in the mixture, X_i is the content in each end-member, and g_i is the fraction of each end-member contributing to the sediment. All end-member proportions are constrained to sum to 1. The parameters used are ^{13}C content, ^{15}N content, organic carbon content (C), total nitrogen content (N), and lignin content (Λ_6; sum of 6 major lignin phenols).

Model ID	No. of Parameters	Parameters				
		^{13}C	^{15}N	C	N	Λ_6
1	2	x	x			
2	2	x		x		
3	2	x			x	
4	2	x				x
5	2		x	x		
6	2		x		x	
7	2		x			x
8	2			x	x	
9	2			x		x
10	2				x	x
11	3	x	x			x
12	3	x		x	x	
13	3	x		x		x
14	3	x			x	x
15	3		x	x	x	
16	3			x	x	x
17	4	x	x	x	x	
18	4	x	x	x		x
19	4	x		x	x	x
20	4		x	x	x	x
21	5	x	x	x	x	x

Table 4.3. For the two and three parameter models, the space bound by the end-members was calculated and standardized to the maximum area. This allowed a comparison between models with different quantities of parameters (as these occupied different dimensional space; e.g. 2 parameter models occupy 2 dimensional space). In addition, the standard deviations of Monte Carlo results from these models were calculated. The correlation between standard deviation in Monte Carlo outputs and the space bound by end-members for each model was calculated three end-members. Correlation coefficients (Pearson's R) and corresponding p-values are reported.

	Pearson's R	**p-value**
C_3 Plant	-0.55	0.029
C_4 Plant	-0.78	0.00041
Marine Diatom	-0.54	0.032

Figure 4.1 Three mixtures were constructed using two end-members; each of the constructed mixtures is identified along the x-axis, with end-members 1 and 2 respectively identified for each of the mixtures. This figure presents a comparison of mass-balance models that solve for g_1, f_1,, and j_1 using OC (left panel) or N (right panel) parameters. g_i represents the fractional contribution of end-member 1 to the constructed mixture—large circles show values of g_i calculated using ^{13}C, and crosses show values of g_i calculated using total (OC or N), isotope content as descriptive parameters in a system of equations, and maintaining the equality constraint; f_i (upright triangles) represents the fractional contribution of parameter signal ($\delta^{13}C$ or $\delta^{15}N$) from end-member 1 to the respective parameter signal in the mixture; upside-down triangles depict values calculated using isotope value and its respective element content (e.g $\delta^{13}C$ and OC content), and satisfying the equality constraint.

Figure 4.2 Comparison of results from Monte Carlo simulations calculating the relative proportion of end-members to a constructed mixture, g_i. The mixture was constructed from equal parts of each of three end-members, and the signal of descriptive parameters within the mixture reflected this contribution. Different mixing models (21; denoted on the x-axis) were used to calculate g_i, with each model using a different combination of parameters that describe the end-members and the mixture. Three normalization schemes were used to standardize the data (none, mean scaling standardization prior to fitting, and z-score standardization prior to fitting). The results from each of the three normalization schemes are shows as a set of three boxes for each of the 21 models. The first in the set of boxes corresponds to data that was not normalized, the second corresponds to data that

was normalized using the mean, and the third corresponds to results from data that had been normalized through z-score standardization.

Figure 4.3 An example used to illustrate how the space defined by end-member parameter values is proportional to the error produced by model estimates. The vertices (circles) indicate standardized parameter-values for each of three end-members for model 1 (a) and model 2 (b). The squares represent the standardized parameter-values of the mixture for reference. The large area defined by parameter values in (a) demonstrate why model 1 was observed to have a small spread in Monte Carlo simulation estimates of g_i (Figure 4.2), while the small area defined by the parameters in (b) explain the large spread in simulation estimates of g_i for model 2.

Figure 4.4 Relationship between the area that is bound by vertices with coordinates corresponding to end-member parameter values, and the error in Monte Carlo simulation estimations of g_i for three end-members.

Figure 4.5 Comparison of fractional contributions of end-members to Altamaha River sediments, as calculated using f_i (top panel) (Dai and Sun, 2007) and recalculated using g_i (bottom panel).

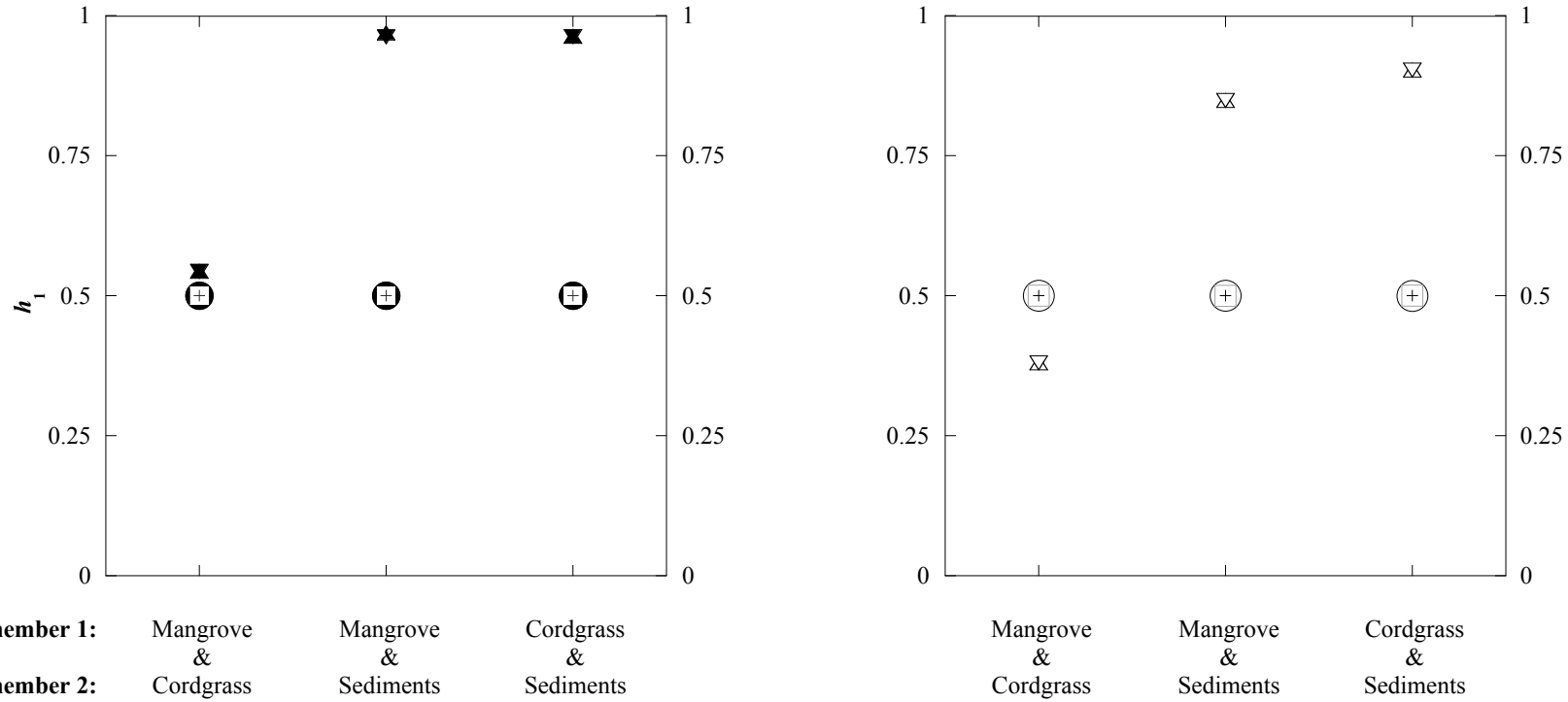
1
0.75
0.5
0.25
0
h_1
Endmember 1: Mangrove & Cordgrass
Mangrove & Sediments
Cordgrass & Sediments
Endmember 2: Mangrove & Cordgrass
Mangrove & Sediments
Cordgrass & Sediments

Figure 4.1

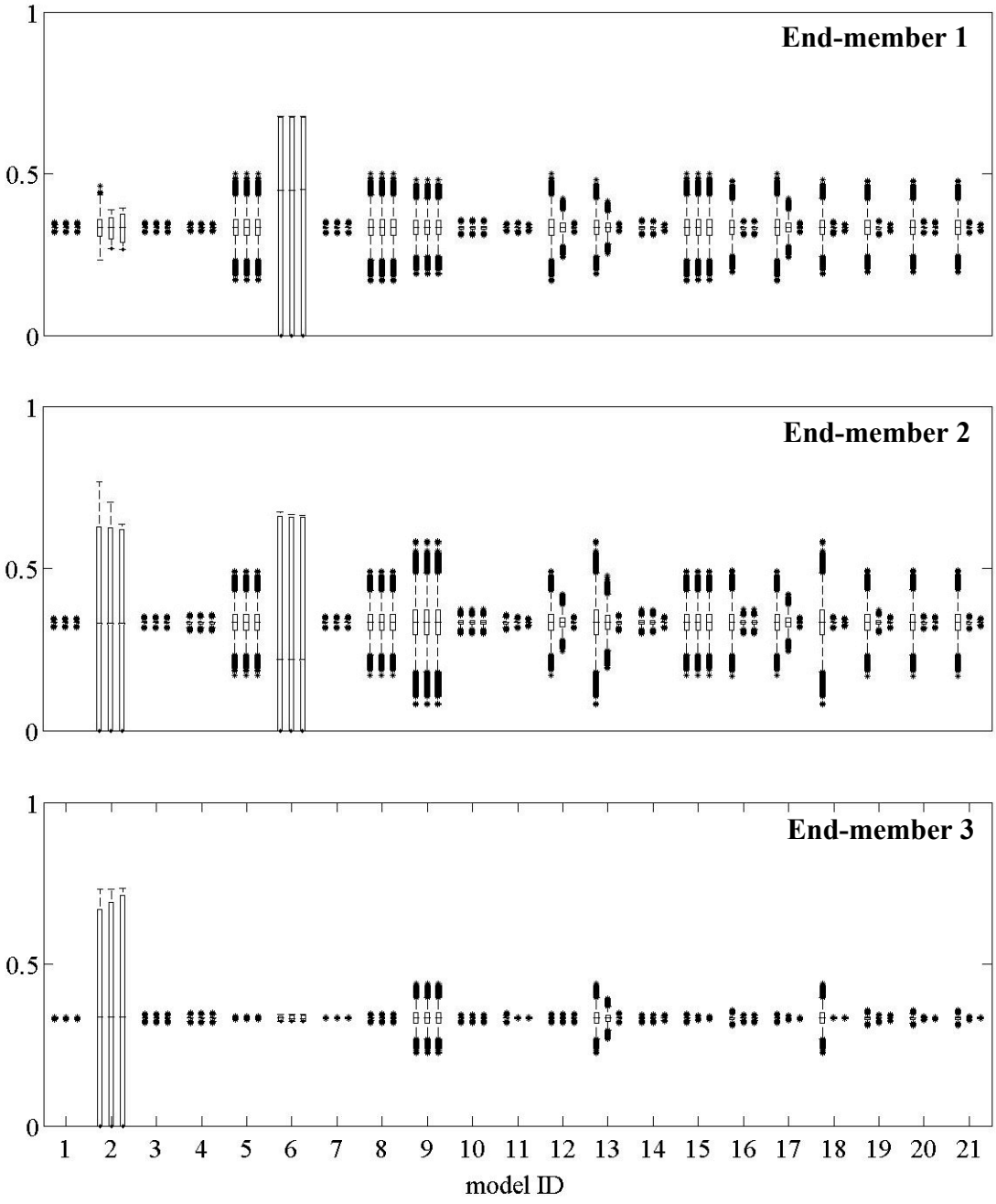
End-member 1
End-member 2
End-member 3
1
0.5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
model ID

Figure 4.2

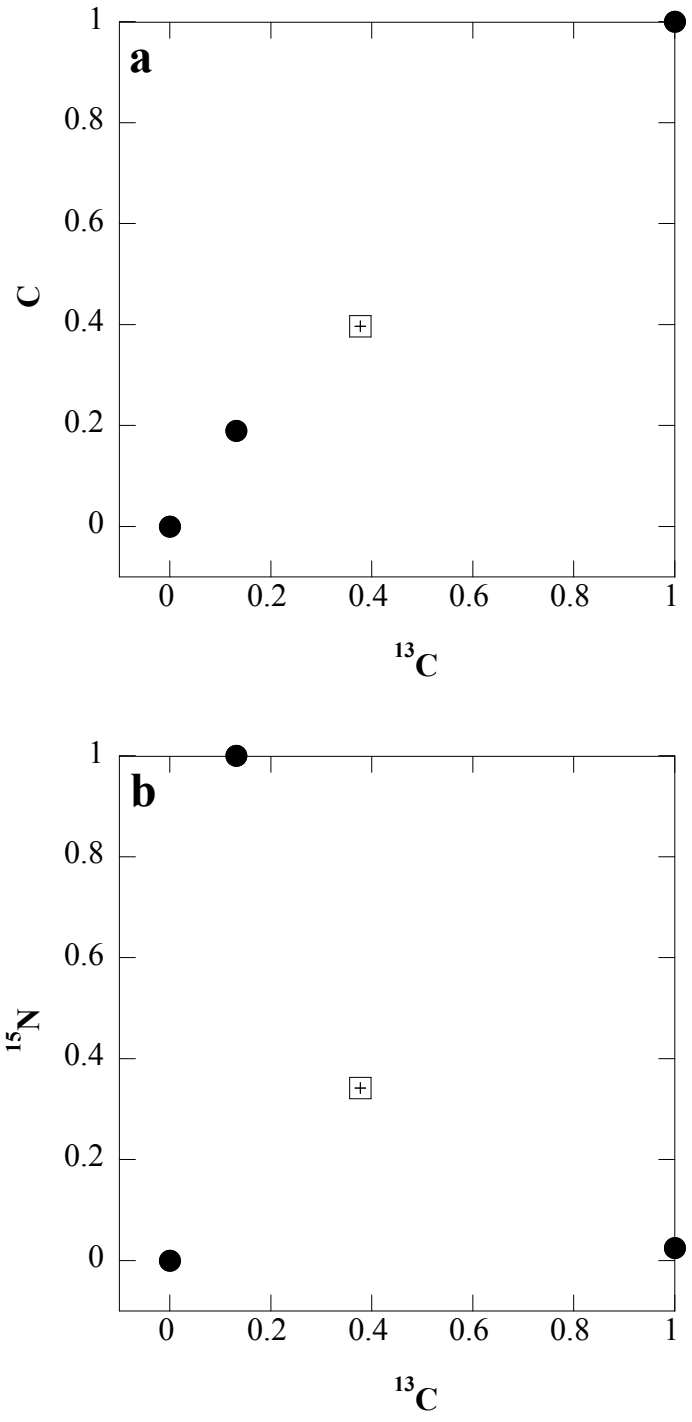

Figure 4.3

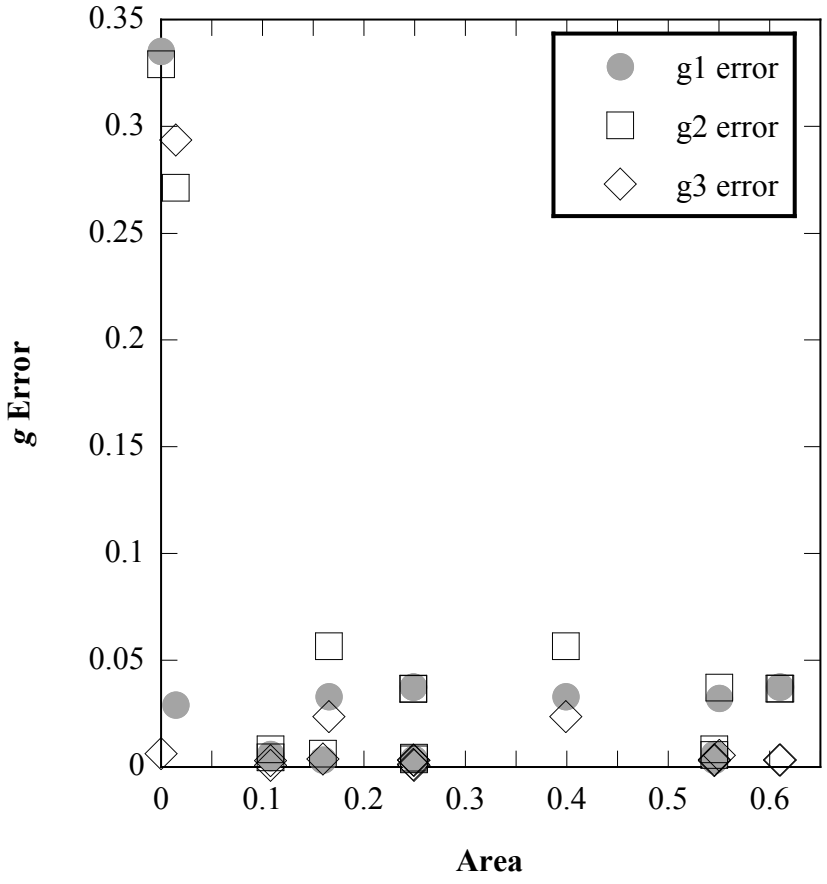
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.1
0.2
0.3
0.4
0.5
0.6
g Error
Area
g1 error
g2 error
g3 error

Figure 4.4

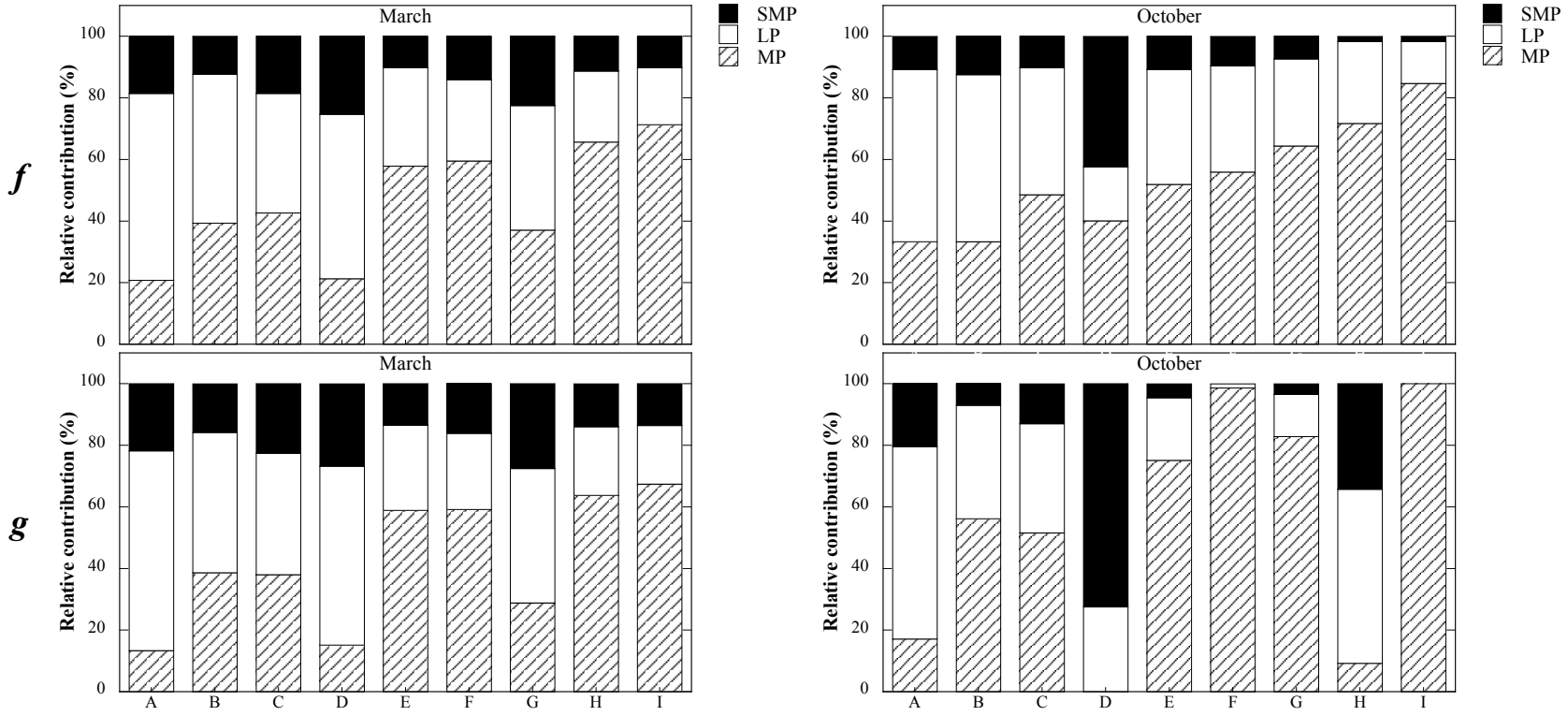
f
g
March
October
SMP
LP
MP
Relative contribution (%)
0
20
40
60
80
100
A
B
C
D
E
F
G
H
I

Figure 4.5

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CHAPTER 5

IDENTIFICATION OF BACTERIAL COMMUNITIES ASSOCIATED WITH VASCULAR PLANT DEGRADATION IN MARSH SEDIMENTS[4]

[4] Loftis, K. M., Sun, M-Y.

Abstract

In marsh sediments, bacteria are important decomposers of organic detritus from marsh plants such as *Spartina alterniflora*. However, the response of bacterial communities to the input of senescent plant litter to sediments is poorly characterized. We conducted in situ incubation experiments in a natural salt marsh environment to investigate how the bacterial communities in surface (0 – 5 cm) and subsurface (>10 cm) sediments vary during decay of *S. alterniflora*. *S. alterniflora* leaves (cut to small pieces) were contained in litter bags and then buried in >10 cm sediments or placed on the sediment surface in a creek of Sapelo Island for incubation over one year. The DNA of litter samples was extracted and the 16S rRNA gene was amplified and sequenced. Two approaches were used to bin sequences and describe the bacterial communities: (1) the operational taxonomic units (OTUs) based on 97% similarity clustering, and (2) the taxonomic classification using RDP. Due to the high level of diversity in the bacterial communities associated with *S. alterniflora* litter that was incubated in surface and subsurface sediments, the 16S rRNA libraries were of insufficient size to confidently quantify the diversity in these communities. However, based on the shape of rarefaction curves, the community on *S. alterniflora* in surface sediments was less diverse than that of subsurface sediments. Classification of sequences at the genus level indicated that members of the genus *Methylobacterium* were dominant on litter in surface sediments. In subsurface sediments, the classified sequences displayed greater diversity.

1. Introduction

Salt marshes are highly productive ecosystems located in coastal areas where physical protection from high-energy waves allows for the establishment of salt tolerant plant species. Salt marshes in the southeastern United States coast are among the most productive ecosystems (Schubauer and Hopkinson, 1984), extending from the state of Virginia to northern Florida. Despite the limited shoreline (160 km), 33% of the total area of salt marshes along the eastern United States is located in Georgia (Wiegert and Freeman, 1990). Salt marshes exhibit low diversity in macrophyte population, rarely containing more than 10 species in the regularly flooded areas (Odum, 1988), and are commonly dominated by a single species (Pomeroy and Wiegert, 1981). Along the east coast marshes of the United States, the low salt marshes are characteristically monotypic, dominated by the cordgrass *Spartina alterniflora*.

In Georgia marshes, 80-96% of primary production is attributed to *S. alterniflora* (Fogel et al., 1989), and approximately 12% attributed to benthic algae and cyanobacteria (Wiegert and Freeman, 1990). *S. alterniflora* occurs either in tall form (1.5 – 2.0 m) near waterways, or in short form (0.3 – 0.6 m) in the higher marshes. As a result of the strenuous physical conditions to which *S. alterniflora* is exposed, *S. alterniflora* creates an extensive network of roots and rhizomes for stability. Belowground production of *S. alterniflora*, then, can be up to 3 times greater than the aboveground production (References in Laffoley and Grimsditch, 2009).

The aboveground biomass of *S. alterniflora* in southeastern United States salt marshes undergoes seasonal variations in abundance, which is linked to cycles of growth and senescence (Schubauer and Hopkinson, 1984). Live biomass increases in the spring

and decreases in the late fall/early winter, with the content of aboveground dead biomass following the inverse pattern. These seasonal patterns control the marsh food web (detritus-based systems), as described by (Teal, 1962). Although *S. alterniflora* serves as a base of the food web in the marsh systems in the eastern United States, only a small fraction of *S. alterniflora* is ingested by herbivores (Teal, 1962). Instead, microbial processes play a more important role in decomposing detritus from the plant *S. alterniflora*. Decomposition of aboveground biomass is controlled by fungal communities when the plants are dead but still standing (Newell et al., 1989). Fungal dominance decreases and the bacterial component increases as the decomposed leaves bend and fell down to the sediment surface (Newell et al., 1989). The leaves of plant are shredded into smaller pieces that are decomposed by detritivores (References in Pennings and Bertness, 2001), and further by sedimentary bacteria.

In the sediments, decomposition of *S. alterniflora* proceeds in three phases: an initial phase marked by a rapid loss in plant biomass, followed by a slower phase of biomass loss, and finally, a phase wherein refractory compounds are dominant, and decomposition essentially plateaus (Valiela et al., 1985).While fungi are the predominant degraders in standing-dead *S. alterniflora*, bacteria mediate degradation of the plant-derived detritus in salt marsh sediments (Benner, Newell, et al., 1984).

Characterizing the microbial community responsible for the decomposition of plant matter and detritus has been an area of interest. T-RFLP and clone library analysis were used to investigate the bacterial assemblages associated with decaying *S. alterniflora* blades (Buchan et al., 2003). During some stages of decay, the microbial community, composed of both fungi and bacteria, was clearly distinguishable (Buchan et

al., 2003). Little is known, however, how bacterial communities respond to *S. alterniflora* at various stages of decay or under different environmental conditions (e.g., surface vs. subsurface conditions in the sediments).

In this study, we explored the bacterial community responses to the inputs of *S. alterniflora* litter in surface and subsurface (> 10 cm depth) marsh sediments. We compared the bacterial communities associated with decaying *S. alterniflora* litter buried in surface sediments and litter buried in subsurface sediments, based on a combined approach of categorizing the sequences into Operational Taxonomic Units (OTUs) and phylotyping. A single taxonomic approach to describe a community is limited by the reference taxonomies (Schloss and Westcott, 2011). Instead, sequence data can be clustered based on similarity between sequences in the community, and binned into OTUs. This method avoids difficulties associated with purely taxonomic methods such as conflicts between different taxonomic outlines, or absence of a similar reference sequence (Schloss and Westcott, 2011). Once sequences were assigned to OTUs, the organism groups were classified, into known taxa when possible, which provided insights into the microbial community structure.

2. Materials and Methods

2.1. Sample description

Sample preparation and incubation was described previously (herein Chapter 2). Briefly, two sets of litterbags containing *Spartina alterniflora* leaves were incubated, in situ, in a small marsh creek in Sapelo Island, coastal Georgia, USA, for 1 year. *S. alterniflora* leaves were collected from the same salt marsh and were rinsed with ultra-

pure water, cut into small fragments (roughly 2mm x 2 mm), and dried. The litterbags were filled with the plant material and were then anchored in the creek sediment environment. One set of litterbags was placed initially at the sediment surface, but were subsequently buried due to sediment accretion (5 cm depth). The second set of litterbags was buried at a depth > 10 cm in the sediments. Litter samples collected at 1 month, 2 month, and 9 month time points were processed for bacterial DNA analysis. Clone libraries for all time points (1, 2, and 9 months) were binned for each setting (surface or subsurface).

2.2. DNA extraction and Polymerase Chain Reaction

DNA from plant leaves within the litterbags and sediment samples was extracted using the MOBIO™ (Carlsbad, CA) Ultrapure Soil DNA extraction kit, following the manufacturer's protocol. Full length bacterial 16S rRNA genes were targeted for amplification with bacteria specific primer B27f (5' AGAGTTTGATCCTGGCTCAG 3') and the universal reverse primer U1492r (5' GGTTACCTTGTTACGACTT 3') (Devereux and Willis, 1995). The PCR master mix (50 μL) was composed of: 2 μL template DNA, 0.5 μL of each primer (100 μmol L^{-1}), 10 μL 5x PCR buffer, 1.25 μL of bovine serum albumin (10 mg/mL), 1 μL of deoxynuceloside triphosphate (10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP), 5 μL of $MgCl_2$ (25 mM), 0.25 μL of Taq DNA polymerase (20 U/μL), and water to a final volume of 50 μL. The PCR cycle began with an initial denaturing of 3 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, annealing at 54 °C for 1 min, elongation at 72 °C for 3 min, and ending with extension at 72 °C for 12 minutes.

2.3. Cloning

PCR products were verified by gel electrophoresis and purified using Qiagen Qia Quick Gel extraction kit, following the manufacturer's protocol. The 16S rRNA gene was ligated into the TOPO® vector (Invitrogen) and transformed into *E. coli* cells using the TOPO® TA cloning kit. Colonies were screened for ampicillin resistance. Sequencing started at the M13 primer within the vector.

2.4. DNA Sequence Processing

Putative chimeras were identified and removed by submitting sequences to the Bellapheron program on the Greengenes website. Sequences were then aligned to the SILVA database. The computer program MOTHUR (Schloss et al., 2009) was used to bin sequences into OTUs defined by 97% sequence similarity. MOTHUR was also used to calculate species richness estimators and to perform hypothesis testing. The number and abundance of OTUs were used to calculate non-parametric richness and evenness indices, including the Chao1 estimator (Chao, 1984), ACE estimator (Chao and Lee, 1992), were calculated by using the method as described in Schloss et al. (2009). Classification of sequences was achieved, when possible, by submitting sequences to the Ribosomal Database Project Classifier (Wang et al., 2007).

3. Results and Discussion

While it is known that bacteria are the predominant degraders of plant matter in marsh sediments, the nature of the bacterial community response to the introduction of plant matter to sediments in these environments is poorly understood. This study set out

to explore how the bacterial sediment community responded to the input of *S. alterniflora* litter to surface and subsurface sediments.

Species richness was evaluated through rarefaction as well as using non-parametric methods. Rarefaction plots the number of types observed, or operational taxonomic units (OTU), against sampling effort. Community diversity and richness impacts the shape of the rarefaction curve, and a curve would asymptote for a thoroughly sampled community. At the point where all OTUs have been identified, increased sampling effort would not yield additional OTUs. A diverse population that is under-sampled, however, more closely resembles a linear trend since the asymptotic section of the curve has not been approached. In this case, additional samples are still yielding new OTUs.

The rarefaction curves for bacterial communities associated with *S. alterniflora* litter incubated in surface and subsurface sediments were calculated for OTUs classified at the 97% similarity cutoff (Figure 5.1). Based on the rarefaction curves, the diversity of the community extracted from litter that was incubated in surface sediments was slightly lower than that of the bacterial community growing on litter incubated in subsurface sediments. The rarefaction curve describing the surface and subsurface communities (Figure 5.1) are characteristic of communities that were not sampled rigorously. This was evident as neither curve reached an asymptote. Additional sampling would be required to produce results at a higher level of confidence.

Another group of methods for assessing community diversity are the non-parametric estimators. These estimators take into account the proportion of organisms that are observed more than once, relative to those that are only observed once. In a

community with low diversity, the probability of observing a single organism more than once will be higher than doing so in a highly diverse population. The Chao1 estimator adjusts the number of observed species by taking into account the number of singleton and doubleton OTUs. This method is better suited for communities with a lower abundance (Chao, 1984). The abundance-based coverage estimator (ACE) accounts for OTUs containing 1-10 individuals (Hughes et al., 2001).

Collector's curves for Chao1 and ACE, much like the rarefaction curve, provide information regarding the level of accuracy in the estimates. Collector's curves produced for the Chao1 and ACE estimators indicated that the estimates for several communities were dependent on the sample sizes. Insufficient sampling was conducted to calculate total community richness (Figure 5.2). This was evident from the shape of the curves, which failed to asymptote.

LIBSHUFF is an implementation that tests for community similarity. Libshuff was utilized to compare bacterial communities associated with *S. alterniflora* litter to communities in sediments. By comparing coverage curves (i.e. how well a sample represents and entire library of sequences), LIBSHUFF estimates the similarity between libraries based on the assumption that two similar libraries will produce similar coverage curves (Singleton et al., 2001). Sequences are randomly shuffled between two libraries of interest and after each shuffling event, the Cramer von Misés test statistic is calculated. This test statistic calculates the distance between the two newly produced coverage curves. If two libraries are highly similar, shuffling sequences between the two samples is still likely to produce two, new, highly similar libraries. Repeating this process n-times allows for the estimation of a p-value. For two libraries, the null hypothesis was rejected,

and a significant difference was found, for an alpha of 0.05. The bacterial community associated with decaying *S. alterniflora* litter in surface sediments was significantly different from that associated with *S. alterniflora* litter in subsurface sediments ($p < 0.0001$).

Unique sequences were submitted to the Ribosomal Database Project classifier for classification. Sequences were classified to the highest taxonomic bootstrap cutoff of 50%. Members of *Methylobacterium* dominated the bacterial community extracted from *S. alterniflora* litter that was incubated in surface sediments (Figure 5.3) accounting for 40% of the clones sequenced from litter incubated in surface-incubated litter. The genus *Methylobacterium* were also fairly prominent (5%) in the bacterial community on *S. alterniflora* incubated in subsurface sediments.

The overriding presence of *Methylobacteria* is notable as members of *Methylobacteria* are characterized as aerobic organisms growing on C-1 compounds, such as methanol and formaldehyde (Kutschera, 2007). These organisms inhabit the rhizosphere and leaf surfaces of plants (Schauer and Kutschera, 2008). During plant growth and cell elongation, methanol is produced and exuded from plant surfaces, where *Methylobacteria* can consume the waste product (Kutschera, 2007). Likewise, damage to plant cells releases these alcohols, along with other organic molecules (Kutschera, 2007). It's reasonable that the *Methylobacteria spp.* present on plant litter were leaf symbionts might therefore be making a living off plant exudates prior to and during incubation. The first step in *S. alterniflora* degradation is a leaching phase (Opsahl and Benner, 1995), and these bacteria may have continued to take advantage of C-1 compounds being released into the sediments during early decomposition. 16S rRNA gene analysis

amplifies the DNA of extant organisms whose DNA has yet to degrade. The dominant presence of *Methylobacterium spp.* may be carry-over from their establishment on the leaf surface of the living plants, with only slow colonization by other bacteria once incubated in sediments.

In surface sediments, a greater portion of the clones was identified at the genus level, with roughly 40% of the classified clones remaining unclassified at this phylogenetic level. On the other hand, roughly 65% of the clones in the subsurface sediments remained unclassified at the genus level. Conversely, the bacterial sequences that could be classified represented 26 different genera on the *S. alterniflora* litter in subsurface sediments, whereas only 12 were represented in surface sediments. Aside from *Methylobacterium spp.*, the predominant, classified genera in subsurface sediments included *Archoleplasma*, *Kosmotoga*, and *Phycisphaera.*

The bacterial community associated with *S. alterniflora* litter that was buried in sediments was highly diverse and, as a result, our sampling efforts were not sufficient to robustly estimate richness. The rarefaction curves indicated that continued sampling would produce new individuals. Further, a confident estimate of the level of diversity in the bacterial communities was also not possible because of the under-sampling and the Chao and ACE collector curves demonstrated that the sampling intensity was insufficient to use these non-parametric estimators. Despite the inability to quantitatively determine the level of diversity of bacterial communities on *S. alterniflora* litter incubated in surface and subsurface sediments, a qualitative comparison was made. Based on the shape of the rarefaction curves, the bacterial community associated with *S. alterniflora* in surface sediments appears to be less diverse than that in subsurface sediments. This trend was

also evident based on species richness when the sequences were classified. Although a smaller percentage of the bacterial community from *S. alterniflora* incubated in subsurface sediments was classifiable at the genus level, a greater number of genera were identified in comparison to that of bacterial communities from *S. alterniflora* litter that was incubated in surface sediments. The low diversity observed for the classified individuals from surface sediment litter was due to the abundance of *Methylobacterium* that were present on this material.

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Figure 5.1. Rarefaction curves for bacterial communities associated with *S. alterniflora* that was incubated in surface (gray) and subsurface (black) sediments. Operational taxonomic units, OTUs, were defined by clustering sequences with a 3%pairwise distance threshold.

Figure 5.2. Collector curves for bacterial communities associated with *S. alterniflora* that was incubated in surface (gray) and subsurface (black) sediments, for the Chao indicator (a), and the ACE indicator (b).

Figure 5.3. Classification of bacterial sequences from *S. alterniflora* litter incubated in surface marsh sediments (5 cm) and subsurface sediments for genera identified at a bootstrap confidence >50%.

a

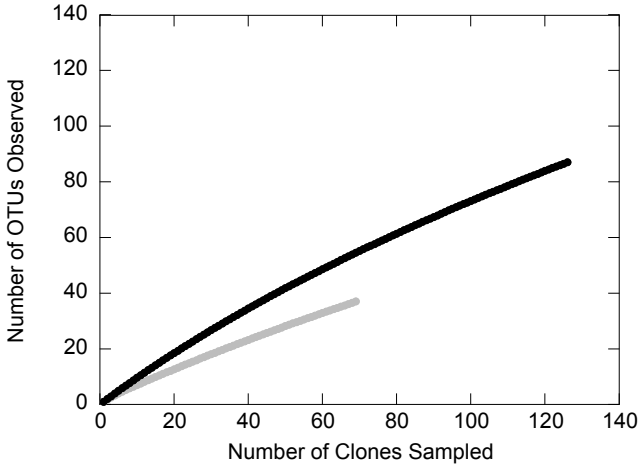
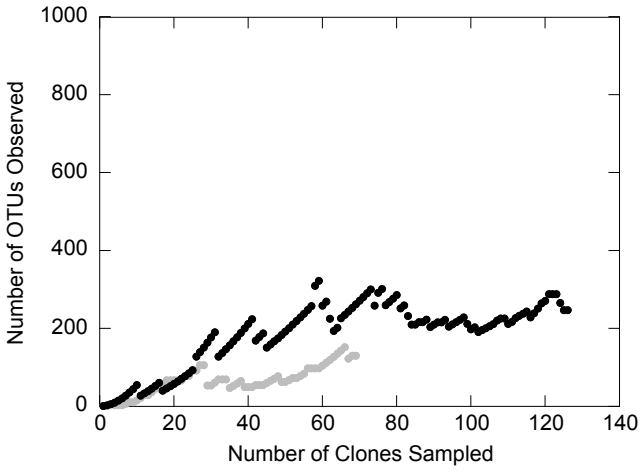

Number of OTUs Observed
Number of Clones Sampled
0
20
40
60
80
100
120
140

Figure 5.1

a


1000
800
600
400
200
0
Number of OTUs Observed
0
20
40
60
80
100
120
140
Number of Clones Sampled

b

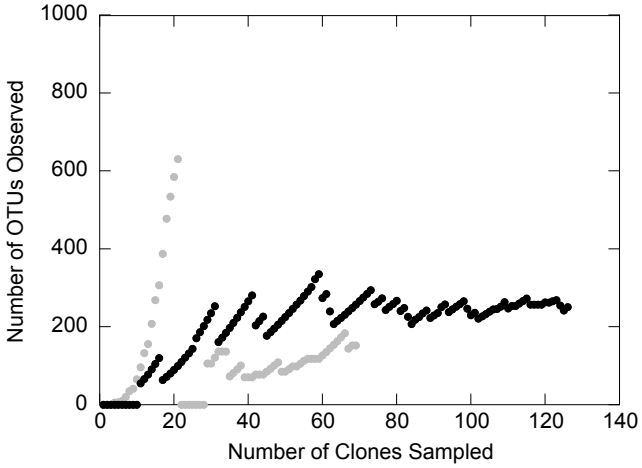

1000
800
600
400
200
0
Number of OTUs Observed
0
20
40
60
80
100
120
140
Number of Clones Sampled

Figure 5.2

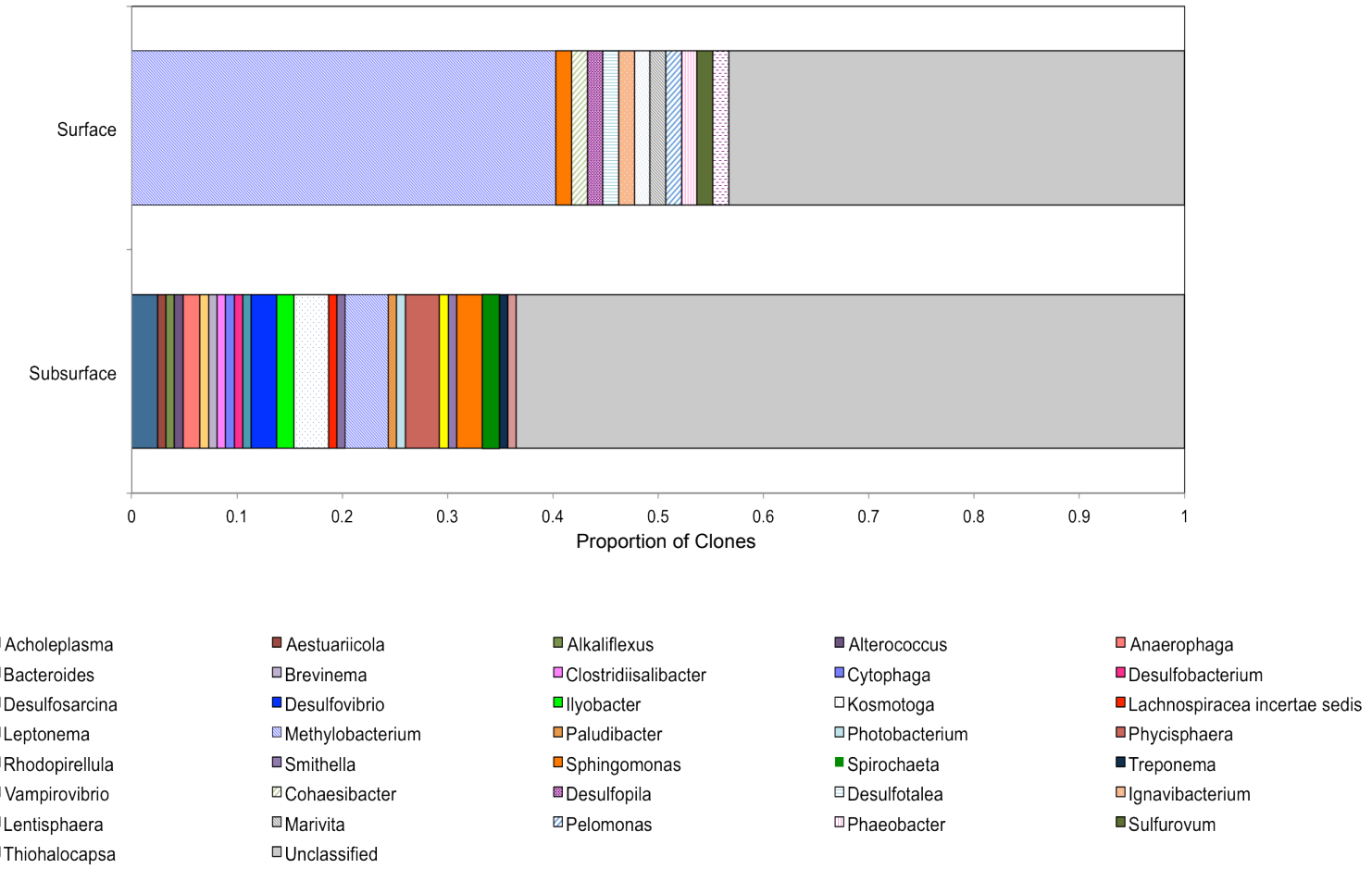

Surface
Subsurface
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Proportion of Clones
Acholeplasma
Aestuariicola
Alkaliflexus
Alterococcus
Anaerophaga
Bacteroides
Brevinema
Clostridiisalibacter
Cytophaga
Desulfobacterium
Desulfosarcina
Desulfovibrio
Ilyobacter
Kosmotoga
Lachnospiracea incertae sedis
Leptonema
Methylobacterium
Paludibacter
Photobacterium
Phycisphaera
Rhodopirellula
Smithella
Sphingomonas
Spirochaeta
Treponema
Vampirovibrio
Cohaesibacter
Desulfopila
Desulfotalea
Ignavibacterium
Lentisphaera
Marivita
Pelomonas
Phaeobacter
Sulfurovum
Thiohalocapsa
Unclassified

Figure 5.3

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CHAPTER 6

SUMMARY

The major objectives of this work were: 1) to determine the temporal variations of marsh plant-derived lignin compositions and their compound-specific isotopic signals during the life-cycle of plants; 2) to assess the effects of plant matter decomposition on the stable carbon isotopes in lignin; 3) to develop a mixing-model that was independent of parameter affects and could best estimate the relative contribution of end-members to a mixture; and 4) to understand how the bacterial community in salt marsh sediments responded to organic matter inputs. In the first study, the temporal variation in $\delta^{13}C$ values of lignin was measured and this information, together with data for lignin parameters, was used to elucidate information on organic carbon dynamics in salt marsh environments (Chapter 2). To better assess the processes governing organic carbon dynamics occurring in salt marsh environments (Chapter 2), the effect of degradation on the $\delta^{13}C$ value of lignin phenols was determined by conducting a series of incubation experiments (Chapter 3). A mixing model capable of employing multiple parameters, including isotope data, was then developed to better estimate the relative contribution of end-members to a mixture (Chapter 4). Finally, we investigated the response of the

ambient bacterial community to organic matter inputs in salt marsh sediments (Chapter 5).

The organic carbon dynamics at salt marsh environments were investigated at two different sites. The $\delta^{13}C$ values of lignin phenols in *S. alterniflora* and *J. roemarianus* were measured. While clear temporal trends were not evident for most lignin phenols in *S. alterniflora*, or for any lignin phenols in *J. roemarianus*, the $\delta^{13}C$ values did vary considerably during the study period. Vanillin exhibited the least variation in carbon isotope composition in both *S. alterniflora* and *J. roemarianus*. Vanillin was found to be the better biomarker, due to the lower variability observed. Carbon dynamics at a creek-bank site were studied and there was evidence of fresh, non-woody plant material deposition during this period. As the season progressed, and the period of low discharge approached, degradation of the plant material that was deposited during the high discharge period was evident. At a high marsh site, during periods of high discharge, chemical parameters indicated the deposition of carbon-rich, non-woody angiosperm tissue.

Different degradation pathways were evident for *S. alterniflora* and *J. roemarianus* incubated in either seawater or sediments. Side-chain oxidation and aromatic ring cleavage were evident during the degradation of *S. alterniflora* in seawater. In addition to side-chain oxidation and aromatic ring cleavage, *J. roemarianus* also underwent demethylation when incubated in seawater. In sediments, *S. alterniflora* lignin degradation occurred primarily through aromatic ring cleavage or solubilization, whereas *J. roemarianus* also underwent side-chain oxidation in surface sediments. The various

lignin degradation pathways employed resulted in varied trends in $\delta^{13}C$ values during decomposition.

A mixing model that uses multiple mass-based parameters was described. We showed that when δ values or elemental ratios are used, the models describe the relative contribution of end-members to parameter signals. We showed that this is not the same thing as describing the relative contribution of end-members to a mixture. This, then, precludes the use of multiple parameters. Instead, a mass dependent parameter model calculates the proportion of end-members to a mixture. Here, multiple parameters can be combined to better constrain the mixture. 21 models were developed, with different parameter combinations and the optimization of parameter choice was investigated. We show, formally, that increased distance between parameter values leads to lower estimation errors.

Finally, the response of bacterial communities to organic matter (*S. alterniflora*) inputs to surface and subsurface sediments at a salt marsh were described. Bacterial communities were characterized using operational taxonomic units (OTUs) and taxonomic classifications. Results showed that the bacterial communities in the litter samples between surface and subsurface sediments responded differently during incubation. The bacterial community on *S. alterniflora* litter in surface sediments did not change significantly over the course of the study. On the other hand, the bacterial community on litter in subsurface sediments did change significantly. The genus *Methylobacterium* was found to be an important member of the community on *S. alterniflora* litter in surface and subsurface sediments. It is possible that these organisms

are epiphytic bacteria that had colonized the leaf litter prior to incubation, and maintained a strong signal throughout the study period.

APPENDIX

COMPARISON of f_{Phillips} AND g

$f_{\text{i,Phillips}}$ approximates (within 1e-4) the mass fractional contribution, g_{i}. The error attributed to calculations of g_i are introduced with assumption A4 (below) is made. $f_{\text{i,Phillips}}$ is a concentration dependent mixing model, and for a two end-member multi-isotope mixing model, takes the form

$$\delta_{\text{mix}} = \frac{f_{1,\text{Phillips}}\delta_1 X_1}{f_{1,\text{Phillips}}X_1 + f_{2,\text{Phillips}}X_2} + \frac{f_{2,\text{Phillips}}\delta_2 X_2}{f_{1,\text{Phillips}}X_1 + f_{2,\text{Phillips}}X_2} \quad \text{(A1)}$$

where $f_{1,\text{Phillips}}$ and $f_{2,\text{Phillips}}$ are the fractional contribution of end-members 1 and 2 respectively. δ are the isotope signals (for a specific element) in the mixture and respective end-members (denoted by the subscripts, mix, 1 and 2, respectively), and X is the elemental concentration.

The generalized isotope mass-balance equation for g_{i} is:

$${}^{x}X_{\text{mix}} = g_1^{x}X_1 + g_2^{x}X_2 \quad \text{(A2)}$$

where ${}^{x}X$ are the mass contents of the isotope of interest in the mixture and two end-members (${}^{x}X_{\text{mix}}$, ${}^{x}X_1$ and ${}^{x}X_2$, respectively). ${}^{x}X$ are calculated from the δ value by $\delta = \left(\frac{R_{\text{i}}}{R_{\text{std}}} - 1\right) \times 1000$, where R_{i} is the ratio of the isotopes in the sample and R_{std} is the ratio of isotopes in the standard. Equation A2, then, can be rearranged to

$$\left[\frac{\left(\frac{\delta_{mix}}{1000}+1\right)R_{std}X_{mix}}{\left(1+\frac{\delta_{mix}R_{std}}{1000}+R_{std}\right)}\right]=g_1\left[\frac{\left(\frac{\delta_1}{1000}+1\right)R_{std}X_1}{\left(1+\frac{\delta_1 R_{std}}{1000}+R_{std}\right)}\right]+g_2\left[\frac{\left(\frac{\delta_2}{1000}+1\right)R_{std}X_2}{\left(1+\frac{\delta_2 R_{std}}{1000}+R_{std}\right)}\right] \quad \text{(A3)}$$

An approximation can be made,

$$\left(1+\frac{\delta_{mix}R_{std}}{1000}+R_{std}\right)\approx\left(1+R_{std}\right) \quad \text{(A4)}$$

This converts Equation A3, after simplification, to

$$\left(\frac{\delta_{mix}}{1000}+1\right)R_{std}X_{mix}=g_1\left(\frac{\delta_1}{1000}+1\right)R_{std}X_1+g_2\left(\frac{\delta_2}{1000}+1\right)R_{std}X_2 \quad \text{(A5)}$$

which can be rearranged to

$$\frac{\delta_{mix}}{1000}X_{mix}=g_1\frac{\delta_1}{1000}X_1+g_2\frac{\delta_2}{1000}X_2-X_{mix}+g_1X_1+g_2X_2 \quad \text{(A6)}$$

The last three terms of Equation A6 cancel out as $X_{mix}=g_1X_1+g_2X_2$. Therefore, Equation A6 can be rearranged and simplified into

$$\delta_{mix}=\frac{g_1\delta_1X_1}{g_1X_1+g_2X_2}+\frac{g_2\delta_2X_2}{g_1X_1+g_2X_2} \quad \text{(A7)}$$

which mirrors Equation A1.