Plant litter composition and stable isotope signatures vary during decomposition in blue carbon ecosystems

J. J. Kelleway · S. M. Trevathan-Tackett · J. Baldock · L. P. Critchley

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Abstract The ratio of isotopes of carbon (13C:12C or δ13C) and nitrogen (15N:14N or δ15N) are common indicators of the flow and storage of organic matter in coastal wetland research. Effective use of these indicators requires quantification and understanding of: (1) the variability of isotope signatures of potential organic matter source materials; and (2) the influence of organic matter decomposition on isotopic signatures. While it is well-established that organic matter characteristics change during the decomposition process, there has been little direct quantification of any concurrent shifts in isotope signatures for coastal detritus. In this study, we addressed this by quantifying: (1) shifts in sample composition using solid-state 13C Nuclear Magnetic Resonance (NMR) spectroscopy; and (2) shifts in δ13C and δ15N signatures of coastal plant tissues from field litterbag experiments. We observed significant shifts in 13C NMR spectra across the course of deployment for all four plant tissues assessed (leaves of mangrove Avicennia marina; branchlets of supratidal tree Casuarina glauca; leaf wrack and roots/rhizomes of the seagrass Zostera muelleri), driven largely by the preferential loss of labile constituents and concentration of more resistant macromolecules, such as lignin and leaf waxes. While there were shifts in isotope ratios for all species, these varied in direction and magnitude among species, tissue type and isotopes. This included δ13C enrichments of up to 3.1‰ and 2.4‰ in leaves of A. marina, and branchlets of C. glauca, respectively, but δ13C depletions of up to 4.0‰ for Z. muelleri. Shifts in δ15N varied among species and tissue types, with few clear temporal patterns. Partial least squares regression analyses showed that some tissue isotope signatures can be reliably predicted on the basis of sample composition (13C NMR spectra), however, multiple inter- and intra-species variations preclude a simple explanation of isotopic signature shifts on the basis of plant-material molecular shifts alone. Further, we cannot preclude the potential influence of microbe-associated organic matter on sample
composition or isotopic signatures. Our findings emphasise the importance of considering decomposition effects on stable isotope signatures in blue carbon ecosystems. Isotope approaches will remain a valuable tool in coastal ecosystem research, but require robust experimental approaches (including appropriate use of decomposed end-members or fractionation correction factors; quantification of microbial organic matter) and quantification of decomposition dynamics for specific plant tissues and environmental settings.

**Keywords** Mangrove · Seagrass · Coastal wetland · Blue carbon · Decay · Organic matter

**Introduction**

Vegetated coastal ecosystems, including seagrass meadows, saltmarshes, mangroves and other tidal forests, are recognised among the most productive ecosystems on earth (Duarte et al. 2013; Krauss et al. 2018; Serrano et al. 2019). This high productivity supports multiple ecosystem services, including: provision of energy and nutrients underpinning coastal and offshore biodiversity and fisheries production (Nagelkerken et al. 2008; Odum & Heald 1975); moderation of biochemical processes, including ocean acidity (Sippo et al. 2016; Wang et al. 2016); and carbon sequestration over centennial to millennial time-scales (i.e. blue carbon) (McLeod et al. 2011; Rogers et al. 2019a). Measuring the flow and/or storage of organic matter (OM) is therefore of importance to our understanding of biochemical connectivity among terrestrial, coastal and marine ecosystems (Abrantes et al. 2015; Alongi et al. 1989; Guest & Connolly 2004; Hemminga et al. 1994). It is also becoming increasingly important in the valuation and accounting of ecosystem services, including the implementation and monitoring of carbon trading and offset projects under blue carbon accounting frameworks (Needelman et al. 2018).

The flow of OM via degradation processes is an important component of carbon cycling budgets (and associated modelling approaches) in blue carbon ecosystems (Arndt et al. 2013; Cebrián et al. 1997; Ouyang et al. 2017). These processes include the decomposition of freshly deposited OM from plants and other autotrophs (e.g. benthic algae)—typically over timescales of hours to months—and the (re)mineralisation of bulk soil or sediment OM, which may extend to centuries or millennia (Goulter & Allaway 1979; Kelleway et al. 2017b; Rogers et al. 2019a). The outcome of these degradation processes can include incorporation of OM into foodwebs, and/or the outward flux of greenhouse gasses through various aerobic and anaerobic pathways and transportation processes (Alongi 2014; Duarte & Cebrian 1996; Maher et al. 2018; Mazumder et al. 2011). The OM that escapes breakdown and transportation has the potential to be sequestered in the substrate and represents an important blue carbon pool (Hyndes et al. 2013; Jennerjahn 2020).

Controls on OM decomposition are diverse and include rate of OM input, temperature, oxygen exposure and the enzymatic capacity of the resident microbial communities (Arndt et al. 2013; Burdige 2007; Cragg et al. 2020). The chemical composition of the plant detritus, in part, influences the breakdown of OM by microbial decomposers (Spivak et al. 2019). For example, the preferential removal of more labile components (e.g. amino acids, polysaccharides) during decomposition leads to the relative enrichment of more recalcitrant OM (e.g. lignin, lipids/waxes) (Benner et al. 1987; Trevathan-Tackett et al. 2017). In addition, a variety of OM sources may accumulate within blue carbon ecosystems, including autochthonous (in-situ production) and allochthonous (external OM) materials of varying recalcitrance (Kelleway et al. 2017a; Kennedy et al. 2004; Trevathan-Tackett et al. 2015, 2017). This complexity emphasises the need for robust biochemical indicators to estimate the source and preservation of various types of OM in blue carbon ecosystems.

The ratio of stable isotopes of carbon (\(^{13}\text{C}:^{12}\text{C}\) or \(\delta^{13}\text{C}\)) and nitrogen (\(^{15}\text{N}:^{14}\text{N}\) or \(\delta^{15}\text{N}\)) are two of the most commonly used indicators of organic matter provenance, transport and cycling in coastal ecosystems. This includes widespread application in food web studies (e.g. Abrantes et al. 2015; Bouillon et al. 2011; Lee 2000; Loneragan et al. 1997; Mazumder et al. 2019) and increasing application (of \(\delta^{13}\text{C}\) particularly) in coastal wetland sedimentary reconstructions (e.g. Johnson et al. 2007; Kelleway et al. 2017a; Rogers et al. 2019b) and blue carbon flux studies (Geraldi et al. 2019; Kennedy et al. 2010; Maher et al. 2013, 2017).

The utility of this approach lies in the fact that different OM sources can have distinct \(\delta^{13}\text{C}\) and/or \(\delta^{15}\text{N}\)
values. Variation in δ^{13}C among broad primary producer groupings (e.g. C3 vs C4 photosynthetic pathways; aquatic vs atmospheric C fixers; algae vs higher plants) has led to use of δ^{13}C as a primary indicator of OM source (Beer 1989; France 1995; Mazumder et al. 2011; Smith & Epstein 1971). Variation in δ^{15}N has been used in many instances as a complement to δ^{13}C for source determination, but is also used, independently, as an indicator of eutrophication (Cole et al. 2004). Predictable isotopic enrichment as prey OM is assimilated by a predator forms the basis for use of δ^{15}N (enrichment of ~3.4‰ commonly used) and δ^{13}C (enrichment of <1‰) as trophic-level indicators (Boecklen et al. 2011; Post 2002).

Reliable apportioning of OM sources and flux using δ^{13}C and/or δ^{15}N values requires: (A) knowledge of potential source values and their variability; (B) sources with sufficiently distinct values; and (C) accounting of any alteration (fractionation) of source values during decomposition (Geraldi et al. 2019; Kelleway et al. 2018). While there continue to be advances regarding (A) (Cernusak et al. 2013; Diefendorf et al. 2010; Farquhar & Richards 1984; Kelleway et al. 2018), and (B) is subject to consideration of specific settings and experimental design; (C) remains an understudied topic for many coastal settings.

Examination of detrital isotope signatures in coastal wetlands has focused largely on the fate of leaf litter of mangroves and seagrass (Dehairs et al. 2000; Dittmar et al. 2006; Kristensen et al. 2008; Rodelli et al. 1984). Isotopic fractionation of seagrass, saltmarsh and mangrove leaves during decomposition has been reported in some studies as low or negligible (i.e. <1 ‰ over 28–168 days of decay, Lanari et al. 2018; Yang et al. 2018; Zieman et al. 1984)). However, larger variations (>2 ‰) can occur, including for δ^{15}N and/or longer-term studies of δ^{13}C (Benner et al. 1991; Fourqurean & Schrlau 2003; Marquez et al. 2017). These varied outcomes highlight our need to better understanding the relationships between plant decomposition dynamics and isotope signatures over a range of coastal settings and timeframes, and the implications for providence and trophic model interpretations.

There are two main methods by which the relationships between plant molecular shifts and isotope signatures have been investigated. The analysis of targeted compounds—chemically extracted from a sample—has been used in ‘compound-specific stable isotope analyses’ from fresh plant and/or detrital sources (e.g. Benner et al. 1987; Dai 2005; Tanner et al. 2010). An alternative approach has been the measurement of identical bulk samples using both isotope ratio and composition analysis methods (e.g. Benner et al. 1990; Dickens et al. 2006; Golchin et al. 1995). In this study, we take the latter approach, characterising compositional shifts via δ^{13}C Nuclear Magnetic Resonance (13C NMR) from field litterbag deployments of four different coastal plant tissues. We then quantify the bulk stable isotope ratios (δ^{13}C and δ^{15}N) of the same samples in order to determine the nature of any relationships between sample OM composition and isotopic indicators (Fig. 1). We test the following hypotheses: (1) there would be significant shifts in OM composition over time for each of the plant tissues tested; (2) there would be significant shifts in isotope values over time among the plant tissues tested; (3) any shifts in isotope ratios could be explained by concurrent shifts in OM composition.

**Methods**

**Litter deployment and collection**

Litter bags provide a standardised method with which to assess in situ OM decomposition (Cummins et al. 1980). In this study, we utilised samples previously subjected to controlled litter deployment and decomposition assessment in the Sydney region, Australia. The species and tissues targeted were leaves of the cosmopolitan mangrove *Avicennia marina*; photosynthetic branchlets of the supratidal tree *Casuarina glauca* (leaves are highly reduced in this genus); and both belowground (root/rhizome) and aboveground (leaf wrack) tissues of the seagrass *Zostera muelleri*.

*A. marina* and *C. glauca* are trees which utilise the C3 photosynthetic pathway, with measurements of live biomass δ^{13}C typically in the range -25 to -31 ‰ (Ball 1988). As with many other seagrass taxa, there remains uncertainty as to the use of C3 and/or C4 pathways by *Z. muelleri* (Kim et al. 2018), with live leaf δ^{13}C measurements in the range -4 to -20 ‰ (Kennedy et al. 2010), potentially explained by its photosynthetic pathway or by the fixation of dissolved CO₂ in the water column, rather than atmospheric CO₂. *C. glauca* has a high nitrogen fixation.
capacity through its root nodule symbiosis with the nitrogen fixing actinobacteria Frankia (Diouf et al. 1995), which may explain the near zero δ\(^{15}\)N values consistently seen in C. glauca plant tissue in its native range (Kurdali & Al-Shamma’A 2009; J. Kelleway, unpublished data). In contrast, it has been shown that δ\(^{15}\)N signatures of plants located lower in the tidal frame are influenced by water column inorganic nitrogen loads (Cole et al. 2004).

The full details of litterbag preparation, deployment, collection and temporal decomposition trends are presented in Critchley et al. (2021) for A. marina and C. glauca, and in Trevathan-Tackett et al. (2020) for Z. muelleri. Briefly, for A. marina and C. glauca, mature leaves that appeared most likely to become detached during storm events were collected from trees in Majors Bay Reserve, Yaralla Bay within Sydney Harbour, New South Wales, Australia (-33.844° S, 151.099° E). Leaves were rinsed, patted of excess moisture, and weighed into ~ 10 g quantities, with wet weights recorded to the nearest 1 mg, then placed in 100 × 150 mm bags made of 1 mm nylon mesh: this mesh size resembles that of loose, naturally entrained leaves in deposition zones (Cummins et al. 1980). These litterbags were deployed on the wetland surface within the intertidal zone. Three replicate litter bags were selected for analysis at each of the following durations after deployment: 7, 21, 42, and 77 days.

For Z. muelleri, fresh, green leaf wrack was collected along the shoreline of Fagans Bay, New South Wales, Australia (33.431°S, 151.321°E). In contrast, root/rhizome material was collected in situ, from living plants. Tissues were washed of sediment and infauna before storage at − 20 °C to stop decay processes before the start of the experiment. The biomass was brought to room temperature, patted of excess moisture and packed into 300 × 300 mm (leaf, 500 g) or 150 × 150 mm (rhizome/root, 60 g) litter bags (1.0 mm polyester mesh; Miami Aqua-culture, Inc., Miami, FL, USA). The seagrass leaf litterbags were deployed at the sediment surface, while the root/rhizome litterbags were buried within the rhizosphere. For both seagrass experiments, three replicate litter bags were selected for analysis at each of the following durations after deployment: 14, 168, 389, and 729 days.

For all species/tissue types, three replicates of initial sample material were washed and dried at 60 °C without being deployed to represent t\(_0\) samples. Upon collection, the litter in the mesh bags was washed of sediments, infauna or algal growth, dried at 60 °C, then kept in cool, dry storage until chemical analyses in the present study (Fig. 1). While care was taken...
to remove non-plant materials, the incorporation of microbial-associated organic matter (including autotrophic fixation) cannot be discounted. All samples were homogenised and ground to a fine powder (Pulvisette 7, Fritsch, Germany) prior to chemical analyses.

**13C NMR**

Solid-state 13C NMR (nuclear magnetic resonance) spectroscopy was used to characterise the molecular composition of carbon within each plant litter sample (Balick et al. 2004). Cross-polarization 13C NMR spectra were acquired using a 200 Avance spectrometer (Bruker Corporation, Billerica, MA, USA) following the instrument specifications, experimental procedures, and spectral processing outlined by Ballock et al. (2013b). Spectra were normalised to a constant total signal intensity, truncated to the 0–200 ppm range, within which the dominant forms of carbon include alkyl C (dominant in 0–45 ppm), N-alkyl/methoxyl C (45–60 ppm), O-alkyl C (60–95 ppm), Di-O-alkyl C (95–110 ppm), aryl C (110–145 ppm), O-aryl C (145–165 ppm), amide/carboxyl C (165–190 ppm), and ketone C (190–200 ppm) (Ballock & Smernik 2002).

**Stable isotope analyses**

δ13C and δ15N were estimated for all samples using an isotope ratio mass spectrometry-elemental analyzer (Thermo DeltaV) at University of Hawaii (HILO). Two quality control reference samples were also included in each analytical run, while a two-point calibration was used to normalise the data using standards USGS40 and USGS41. Results are accurate to 0.2‰ of measured δ13C values (reported relative to Vienna Pee Dee Belemnite) and 0.2‰ of measured δ15N values (reported relative to air).

**Data analysis**

Multifactorial permutational analyses of variance (PERMANOVAs) were used to investigate differences among 13C NMR spectra across the course of the decomposition experiments. Separate PERMANOVAs were completed for each tissue type, while pairwise comparisons were used to identify differences among time points. Monte Carlo-approximated P values (P(MC)) were used to interpret comparisons with low numbers of unique permutations (i.e. < 10). These analyses were based on Euclidean distance resemblance matrices and were performed using PRIMER version 6 with PERMANOVA add-on (PRIMER-E, Auckland, New Zealand).

Principal components analysis (PCA) was performed using the normalised 13C NMR spectra to: (1) identify temporal trends in composition among samples over the course of the decomposition experiments; and (2) define the 13C NMR spectral components most important to differentiating the samples. Loadings were plotted for the first two principal components to assist in the latter and to guide interpretation of differences in composition among samples.

Variations in stable isotope values (δ13C and δ15N) were assessed in several ways, with separate analyses conducted in all instances for each of the four tissue types and two isotopes analysed. First, isotope values were regressed against deployment time, with linear or exponential decay relationships fitted as appropriate. Second, one-way analyses of variance (ANOVA) were performed to test for significant variations in isotope values between all of the sampling periods, including original materials (t0). Welch’s F-tests were used where the homogeneity of variance assumption was not met (Delacre et al. 2019), while Bonferroni (ANOVAs) and Games-Howell (Welch tests) post-hoc comparisons were also undertaken.

Partial least-squares regression analysis (PLSR) has been widely used to predict soil and plant carbon concentrations and/or carbon fractions on the basis of spectroscopic data (Ballock et al. 2013a; Hayes et al. 2017; Wold et al. 2001). In this study, we used PLSR to test whether sample stable isotope ratios (δ13C and δ15N) could be predicted from 13C NMR spectra. PLSR is useful in this instance as it extracts a set of orthogonal factors from potentially numerous and highly-correlated predictor variables (i.e. NMR spectra) and response variables (i.e. stable isotope signatures). Separate analyses were conducted for each tissue type and each isotope ratio, whereby all samples across the time series were used to calibrate models, and also to validate models using a leave-one-out cross-validation approach.
Results

OM composition

For *Avicennia marina* leaf samples, there were significant shifts in spectral signatures over the course of the litter deployment period (P(MC) = 0.001; Table S1). There were increases in spectral intensity in the region 20–40 ppm, and at 150–155 ppm, with decreases at 72–76 ppm, 113–120 ppm and 142–150 ppm, and an initial decrease only at 56 ppm (methoxy C) (Fig. 2a). There were significant pairwise differences between t₀ and t₇ spectra, as well as these two sampling events versus the three later events (pairwise comparison P(MC) values < 0.05), consistent with their distribution along PC1 (Table S1; Figure S1). There were no significant differences among t₂₁, t₄₂ and t₇₇ despite some separation along PC2 (Table S1; Figure S1).

For *Casuarina glauca* branchlets, shifts in ¹³C NMR spectra over the course of deployment included increases in spectral intensity at 142–147 ppm and 150–155 ppm (both in common with *A. marina*), but opposing trends to *A. marina* of decreasing intensity at 56 ppm (methoxy C) and in the range 20–40 ppm (Fig. 2b). There was a significant difference between t₀ and all sampling events after t₇ (pairwise comparison P(MC) values < 0.05), with t₀ through t₂₁ separated across PC2, followed by later separation across PC1 (t₄₂, t₇₇; Table S1; Figure S1b).

For *Zostera muelleri* leaf wrack, significant differences among time periods (P(MC) = 0.0016) were driven by longer-term shifts in ¹³C NMR spectra (Fig. 3a). That is, pairwise comparisons showed differences were only significantly different at P(MC) < 0.05 from t₀ by the final sampling period (t₇₉), though all other comparisons to t₀ had P(MC) values < 0.1 (Table S1) and some separation among the first two principle components (Figure S2a).

![Fig. 2](image)

Mean ¹³C NMR spectra of decomposition samples for *Avicennia marina* (a) and *Casuarina glauca* (b) colour coded by collection time (replicate n = 3). Arrows represent the direction of shifts in spectra across the course of the experimental period (blue and red arrows represent shifts in a similar or opposite direction, respectively, to that observed for *A. marina*). Lower plots (c, d) display the mean relative contribution of variation in ¹³C NMR spectra toward the prediction of δ¹³C as determined through partial least squares regression. This contribution was calculated by multiplying the PLS regression β-coefficient (averaged across all samples for a species) by the spectral intensity (averaged across all samples for a species) for each unit of chemical shift (ppm). The size of peaks and troughs in c and d therefore signify relative importance to the predictive models.
Similarly, for Z. muelleri root/rhizome, significant overall difference was also driven by longer term shifts in $^{13}$C NMR spectra—in this case significant differences only between $t_{14}$ and $t_{729}$ and between $t_{168}$ and $t_{729}$ (Table S1).

Inspection of $^{13}$C NMR spectra revealed increases over time for both seagrass tissue types in intensity in the range 20–40 ppm, and decreases over time at 72–76 ppm (Fig. 3a, b)—both in common with A. marina. In contrast to A. marina, there were broad increases in the range 115–160 ppm (including peaks at 128 and 150 ppm) for late-stage samples. There were broad similarities in magnitude and direction of spectral shifts for leaf wrack and root/rhizome over the experimental period (Fig. 3a, b), with the first principal component capturing similar variability (Figure S2a, b), with broadly similar—though inverse—coefficient plots (Figure S2c, d).

Isotopic signatures

For A. marina and C. glauca, $\delta^{13}$C signatures experienced shifts best described by exponential decay models (Fig. 4a, b). That is, there was a significant enrichment in A. marina leaf $\delta^{13}$C between deployment ($t_0$) and the first collection ($t_7$) (mean shift = 2.8‰; $P < 0.01$), but little change through the remainder of the experimental period (Fig. 4a; Fig. S3a). Similarly, a significant, though smaller initial shift was also observed for branchlets of the C. glauca (mean shift = 1.6‰; $P < 0.001$), as part of an exponential decay (Adj. $R^2 = 0.89$; Fig. 4b; Fig. S3d).

In contrast, fractionation of Z. muelleri occurred in the opposite direction (i.e. a depletion in $\delta^{13}$C), exhibiting a strong relationship for root/rhizome (Adj. $R^2 = 0.89$; $P < 0.001$; Fig. 4d; Table S2; Figure S4d), and a weak linear relationship for leaf wrack (Adj. $R^2 = ...$)

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**Fig. 3.** $^{13}$C NMR spectra of decomposition samples for Zostera muelleri leaf wrack (a) and root/rhizome (b) colour coded by collection time (replicate $n = 3$). Arrows represent the direction of shifts in spectra across the course of the experimental period (red arrows represent shifts in an opposite direction to that observed for A. marina). Lower plots (c, d) display the mean relative contribution of variation in $^{13}$C NMR spectra toward the prediction of $\delta^{13}$C as determined through partial least squares regression. This contribution was calculated by multiplying the PLS regression $\beta$-coefficient (averaged across all samples for a species) by the spectral intensity (averaged across all samples for a species) for each unit of chemical shift (ppm). The size of peaks and troughs in c and d therefore signify relative importance to the predictive models.
R² = 0.44; Welch P = 0.044; Fig. 4c; Table S3; Figure S4a).

For δ¹⁵N, there were significant overall differences among sampling periods for all sample types, except *Z. muelleri* root/rhizome (Tables S2, S3). Pairwise comparisons of sampling periods showed initial (t₀ to t₇) shifts of a similar magnitude observed in δ¹⁵N between *A. marina* (mean shift = 2.2‰; P = 0.015) and *C. glauca* (mean shift = -2.0‰; P = 0.003), however these were in opposite directions to one another (Fig. 5). After t₇ there were no clear trends for either species, with values moderating between the t₀ and t₇ values. There was a significant depletion in δ¹⁵N for *Z. muelleri* leaf wrack (P < 0.001). While this returned a moderate linear fit (Adj. R² = 0.60), separation between fresh and short-term deployment and longer-term deployment suggests non-linear trends (Fig. 5c).

Predicting isotope ratios

The analytical measurements of δ¹³C and δ¹⁵N ratios were compared against predictions of these ratios based upon their ¹³C NMR spectrum (Fig. 6). Prediction capacity was greatest for δ¹³C of *C. glauca* branchlets (validation R² = 0.91) with a prediction RMSE of 0.29‰, followed by δ¹³C of *A. marina* leaves (R² = 0.74). Prediction capacity was lower, though moderate, for *Z. muelleri* δ¹⁵C. Although calibration data returned moderate to high R² values for all δ¹⁵N tests, weaker relationships for validation values suggest a low predictive capacity, except for *Z.
muelleri leaf wrack where predictive capacity appears moderate ($R^2 = 0.69$; prediction RMSE = 0.6‰).

Inspection of the $\delta^{13}$C PLSR $\beta$-coefficient plots reveals several differences among taxa. While there were significant peaks or troughs across the range 65–85 ppm for all taxa, the direction and specific position of these varied among the tree versus seagrass samples. That is, there were broad troughs (negative coefficients) for A. marina (Fig. 2c) and C. glauca (Fig. 2d) in this range, but a series of peaks (positive coefficients) at 69–70 and 74–82 ppm and troughs at 65 and 72 ppm for the two seagrass tissues (Fig. 3c, d). The tree tissues also had peaks centred at 105 ppm (larger for C. glauca relative to A. marina), whereas the seagrass tissues had narrow troughs here. Outside these spectral ranges, A. marina alone exhibited broad troughs at 52–56 ppm and 25–35 ppm, the latter of which was contrasted by a peak for C. glauca (31–35 ppm). The relative $\beta$-coefficient spectra for the two seagrass samples were near identical. The $\delta^{15}$N $\beta$-coefficient plot for Z. muelleri leaf wrack shows peaks and troughs at 68, 76, 105, 174 and 177 ppm (Figure S5).

**Discussion**

**OM composition changes during decomposition**

In this study, we observed statistically significant temporal shifts in the carbon composition of each of the four coastal wetland plant tissues assessed, thereby confirming our first hypothesis. The timing and nature of these shifts, however, were variable amongst the tissue types assessed (Figs. 2 and 3; Table S1), precluding a simple, universal model of...
litter decomposition. For the leaves of the mangrove and branchlets of the swamp oak, compositional shifts were evident early in the sampling period (by $t_7$ for and $t_{21}$, respectively), consistent with the depletion of O-alkyl carbon (namely cellulose at 72–76 ppm), and shifts in O-aryl carbon (142–147 and 150–155 ppm). For these species, early diagenesis was followed by further depletion of O-alkyl and di-O-alkyl carbon (95–110 ppm), consistent with depletion of carbohydrates (Baldock & Smernik 2002) and a corresponding relative enrichment in lignin (enrichment at 142–147 ppm and 56 ppm, albeit with potential lignin depletion at 150–155 ppm) through the remainder of deployment. In contrast, for the seagrass *Zostera muelleri*, there were no consistent differences between $t_0$ and the first sampling period ($t_{14}$). There were longer-term shifts for *Z. muelleri*, however, including depletions diagnostic of carbohydrates (e.g. cellulose at 72–76 ppm and 105 ppm), and enrichments across ranges indicative of lignin, protein and/or lipid carbon (20–60 ppm and 120–160 ppm). While our experimental methods do not preclude the incorporation of 'new' OM from microbial autotrophs or heterotrophs, these broad compositional shifts of carbohydrate loss and relative enrichment of lignin (and/or other components) are consistent with a body of research on plant litter decomposition. This includes mangrove and seagrass detritus studies that have recorded mineralisation of polysaccharides at 2 to 10 times greater than lignin components (Berner et al. 1990, 1984; Trevathan-Tackett et al. 2017). We therefore conclude that the compositional shifts observed are largely related to decomposition of the original vascular plant tissues.

Some of the variation between tissue types is likely explained by the different experimental conditions used in the two decomposition experiments. First, the seagrass sampling regime captured a longer period, encompassing post-leaching remineralisation and stable phases (Trevathan-Tackett et al. 2020) not captured in the shorter experiment (Critchley et al. 2021), though these do not explain the early-phase differences. The timescales under which we observe OM degradation influences the factors exerting control over OM turnover (Burdige 2007; Trevathan-Tackett et al. 2020). For example, decomposition studies in BC ecosystem typically capture the early stages of OM diagenesis (i.e. within the first 1–2 years or

![Fig. 6](image-url) Measured vs predicted plots of stable isotope ratios ($\delta^{13}C$ in top panel, $\delta^{15}N$ in lower panel) measured with isotope-ratio mass spectrometry against model-predicted values from partial least square regression analyses of $^{13}C$ NMR spectra. For each measured sample there is a pair of predicted data—hollow symbols and dashed lines represent calibration predictions; filled red samples and solid lines represent leave-one-out validation predictions. Linear relationships are provided by sample type: *Avicennia marina* leaf (a, b), *Casuarina glauca* branchlet (c, d), *Zostera muelleri* leaf wrack (e, f) and *Zostera muelleri* root/rhizome (g, h)
less). During this time, leaching is followed by the early stages of enzymatic breakdown of the detritus by resident microbial populations. Long-term turnover of detritus (> 5 years) is difficult to capture within decomposition studies, yet we know this detrital OM sequestration occurs, as evidenced through biomarker studies, such as stable isotopes (Kennedy et al. 2010).

The varied inundation conditions for seagrass (subtidal settings for leaf wrack, and within the rhizosphere for root/rhizome) compared to A. marina and C. glauca (exposed to sub-daily wetting and drying cycles on the wetland surface in the mid intertidal zone) were likely important. Less oxic conditions in the seagrass deployment would explain slower decomposition (Wang et al. 2019). A lack of significant differences in composition of t0 versus all collections other than t129 for Z. muelleri leaves, might also be explained by our use of leaf wrack from t0 which would have undergone some early leaching and/or diagenesis prior to collection (Trevathan-Tackett et al. 2020), thereby missing potentially significant early changes in macromolecular content and/or isotopic signatures. The presence of microbe-associated OM (either autotrophs or heterotrophs) on this wrack material also cannot be discounted.

There were, however, several differences in decomposition amongst tissues subjected to identical deployment regimes. For example, the 13C NMR spectra for A. marina differed from C. glauca in two key ways. First, the A. marina spectra showed an enrichment in alkyl carbon (20–40 ppm) (Fig. 2a), which was important to the separation of samples collected early (t0 and t1) versus later (t3 onwards) in the experiment (Figure S1a, c). In contrast, C. glauca exhibited a depletion in this region (Fig. 2b; Figure S1b, d). The second difference between these species was the initial and rapid depletion at 56 ppm (methoxy C) for A. marina, versus a later enrichment here for C. glauca. These differences might be attributed to a higher proportion of lignin (methoxy C) loss from mangrove leaves, but long-term preservation of mangrove leaf waxes (alkyl C) (Koch et al. 2011), relative to the highly-reduced leaf and more woody structure of C. glauca branchlets. These among-taxa differences highlight the significant role of the original material’s chemistry in driving decomposition dynamics (Trevathan-Tackett et al. 2017), and the importance of taxon-specific assessment in understanding plant tissue diagenesis.

**Shifts in δ13C and δ15N during decomposition**

The δ13C and δ15N values we report for t0 samples are within the range previously reported for fresh materials (or recent seagrass wrack) of these species in the study regions (Kelleway et al. 2018, 2017b; Trevathan-Tackett et al. 2020). During the decomposition phase, however, we observed large shifts in mean δ13C values of up to 3.1‰ enrichment (A. marina leaf) to 4.0‰ depletion (Z. muelleri root/rhizome) (Table 1). These maximum δ13C shifts are larger than most previous reports for blue carbon ecosystems (Table 1) and larger than most estimates in the terrestrial literature (Fernandez et al. 2003; Osono et al. 2008; Schweizer et al. 1999). As with 13C NMR spectra, there was division in the response of isotopic signatures between the two tree species deployments compared to the two seagrass tissue deployments. Most importantly, our comparison revealed a significant enrichment during in δ13C during decomposition for both tree species, contrasted by significant depletions in δ13C for both seagrass tissues. While we partially accept our hypothesis that there would be significant shifts in isotope signatures over time, our findings for δ13C, and the temporally variable nature of δ15N signatures, highlights a more complex circumstance.

The opposing direction of change between A. marina / C. glauca (δ13C enrichment) and the seagrass tissues (δ13C depletion) is an important outcome of this study, which we explore in the context of chemical composition changes in the following section. Regardless of direction, the magnitude of the shifts have important implications for the design and interpretation of stable isotope studies. Given that many food web and organic matter burial studies interpret shifts of 1‰ or less as indicative of retention of an original source material, the variations we observed during decomposition—rapid in some cases (Figure S3)—suggest quantification of diagenesis effects and/or use of appropriate litter end-members may be necessary (Dai et al. 2009). The exponential decay models for A. marina and C. glauca (Fig. 4a,b) and the approximately linear relationship for Z. muelleri roots/rhizomes (Fig. 4d) of the stable isotope signatures over time provide a starting point for improved estimates of these litter contributions to food webs, organic matter burial, among other applications. Further work, however, is required.
Table 1  Summary of maximum shifts and experimental conditions of mean stable isotope values relative to starting material (t₀)

| Species                  | Tissue          | Deployment duration (d) | Experiment setting | Shift (%ε) relative to t₀ | References                                      |
|--------------------------|-----------------|-------------------------|--------------------|---------------------------|------------------------------------------------|
|                          |                 |                         |                    | δ¹³C | δ¹⁵N          |                                                   |
| Mangrove                 |                 |                         |                    |                 |                                                 |
| Avicennia marina         | Leaf            | 77                      | Field              | 3.1  | 2.2           | This study*                                      |
| Avicennia marina         | Leaf, green     | 168                     | Field              | 2.4  | -             | Yang et al. (2018)                              |
| Avicennia alba           | Leaf, yellow    | 92                      | Field              | -0.2 | -0.7          | Nordhaus et al. (2017)                          |
| Aegiceras corniculatum   | Leaf, yellow    | 92                      | Field              | -0.8 | -0.4          | Nordhaus et al. (2017)                          |
| Ceriops decandra         | Leaf, yellow    | 92                      | Field              | 0.1  | -2.1          | Nordhaus et al. (2017)                          |
| Sonneratia caseolaris    | Leaf, yellow    | 92                      | Field              | 0.6  | -0.3          | Nordhaus et al. (2017)                          |
| Rhizophora apiculata     | Leaf, yellow    | 92                      | Field              | -1.3 | -0.5          | Nordhaus et al. (2017)                          |
| Rhizophora mangle        | Leaf, yellow    | 348                     | Field              | 2.0  | -2.0          | Fourqurean and Schrlau (2003)                    |
| Bruguiera gymnorrhiza    | Leaf, green     | 168                     | Field              | -1.4 | -1.4          | Yang et al. (2018)                              |
| Seagrass                 |                 |                         |                    |                 |                                                 |
| Zostera muelleri         | Leaf wrack      | 729                     | Field              | n.s. | -2.0          | This study*                                     |
| Zostera muelleri         | Root/rhizome    | 729                     | Field              | -4.0 | n.s           | This study*                                     |
| Halodule wrightii        | Leaf            | 28                      | Laboratory         | -0.3 | 0.4           | Macko et al. (1994)                             |
| Thalassia testudinum     | Leaf, green     | 77                      | Field              | 1.0  | -             | Delgado et al. (2017)                           |
| Thalassia testudinum     | Rhizome         | 77                      | Field              | 0.0  | -             | Delgado et al. (2017)                           |
| Thalassia testudinum     | Leaf, green     | 348                     | Field              | -2.0 | >2            | Fourqurean and Schrlau (2003)                    |
| Thalassia testudinum     | Rhizome         | 348                     | Field              | 0.0  | 0.0           | Fourqurean and Schrlau (2003)                    |
| Saltmarsh                |                 |                         |                    |                 |                                                 |
| Spartina alterniflora    | Root/rhizome    | 550                     | Field              | 1.0  | 2.0           | Benner et al. (1991)                            |
| Spartina alterniflora    | Leaf            | 60                      | Laboratory         | 1.99 | 3.54          | Dai (2005)                                      |
| Spartina densiflora      | Leaf, green     | 63                      | Field              | -1.2 | 1             | Lanari et al. (2018)                            |
| Scirpus maritimus        | Leaf, green     | 63                      | Field              | -0.3 | -0.6          | Lanari et al. (2018)                            |
| Supratidal forest        |                 |                         |                    |                 |                                                 |
| Casuarina glauca         | Branchlet       | 77                      | Field              | 2.4  | -2.0          | This study*                                     |

*Values from this study are the maximum shift in mean value from t₀ observed throughout the deployment period. No values are presented (n.s.) where no significant shifts were identified (alpha level P < 0.05) from t₀ value during the experimental period

to determine the nature of these relationships over longer timeframes (especially for A. marina and C. glauca), and under varied decomposition settings.

Despite significant δ¹⁵N differences among sampling periods for three out of four tissues types (Tables S2 & S3), there were few clear temporal patterns in δ¹⁵N throughout the litter deployments. While a moderate relationship (R² = 0.6) was fitted for Z. muelleri leaf wrack, we advise against use of this as an isotope decay factor, given the potential for non-linearity here, as well as the variability in δ¹⁵N across all sample types. One potential explanation for variability of δ¹⁵N could be temporal variability in nitrogen loads, which vary in relation to catchment inputs (Birch et al. 2010; Oeurng et al. 2010). Benthic sediment organic matter—such as live estuarine plants—has been shown to hold δ¹⁵N signatures reflective of anthropogenic nitrogen loads in the local water column (Mazumder et al. 2015). This, however, does not explain the differential shifts in δ¹⁵N by A. marina litter (enrichment of up to 2.2‰) versus C. glauca (depletion of up to 2.0‰) in otherwise identical, adjacent experimental plots. Variation of δ¹⁵N signatures during the decomposition process may also be influenced by the source and process of external N uptake from the environment by the detritus-associated microbiota to assist in the breakdown process (N immobilisation; Benner et al. 1991; Bouillon et al.)
et al. 1995). These differences point to the importance of the original plant material, and the potential for differential responses of microbial breakdown and N immobilisation during the decay process among different plant substrates (Table 1), which otherwise inhabit similar physiological niches. For $\delta^{15}N$, these taxon-specific variations, combined with temporal variations, highlight the need for understanding drivers of $\delta^{13}C$ variation in coastal wetland litters before this parameter can be reliably used in source attribution studies.

Relationships between organic matter composition and stable isotope signatures

In this study, we’ve been able to quantify relationships between bulk sample composition and selected stable isotope signatures. Generally, we found much stronger relationships between $^{13}C$ NMR spectra and $\delta^{13}C$, rather than with $\delta^{15}N$. This is not particularly surprising, given that our composition method specifically assesses carbon rather than nitrogen atoms, though $^{13}C$ NMR is useful for the inference of non-carbon organic matter, especially via carbon associated with plant amide, nitrile and nitro compounds.

Much of the literature on isotopic signature changes during plant material decomposition is framed around differences in the $\delta^{13}C$ signatures of cellulose and lignin. Various studies report cellulose as more enriched in $^{13}C$ than lignin (Fernandez et al. 2003; Loader et al. 2003); more depleted than lignin (Gori et al. 2013); or opposing $\delta^{13}C$ trends during lignin accumulation among C3 and C4 plants (Wedin et al. 1995). While our interpretation of $^{13}C$ NMR spectra emphasises a loss of carbohydrate carbon during decomposition, and a relative accumulation of lignin and other resistant molecules (see section above), the influence of these changes on samples $\delta^{13}C$ is inconsistent among samples (see lower panels of Figs. 2 and 3).

First, we see the loss of O-alkyl carbon (65–85 ppm; diagnostic of carbohydrates including cellulose) being strongly associated an enrichment in $\delta^{13}C$ (signified by a negative coefficient operating on a negative $\delta$ value) for A. marina and C. glauca, versus a net depletion effect in the Z. muelleri tissues. An opposing contrast is seen in disparate depletion effect (C. glauca, A. marina) versus enrichment effect (Z. muelleri) centred at 105 ppm—which is also diagnostic of cellulose. Second, inspection of the $\beta$-coefficients for C. glauca (the species for which lignin accumulation was most apparent) shows a similarly complex circumstance for lignin. That is, while $\beta$-coefficients peaks at 150–155 (O-aryl carbon) and 56 ppm (methoxy carbon) suggest the accumulation of some lignin components results in an enriched $\delta^{13}C$ prediction, the loss of carbon in the 142–147 ppm range (also diagnostic of lignin) did not appear influential to $\delta^{13}C$. Together, these outcomes suggest that although $\delta^{13}C$ are associated with species-specific changes in lignin and cellulose contents and composition, these shifts are complex, and potentially divergent among taxa of diverse initial $\delta^{13}C$ signatures.

There are multiple biochemical processes that may contribute to concurrent and related shifts we observed in litter composition and $\delta^{13}C$ signature of each tissue type. In addition to the changes in the original plant material during decomposition, there is also the potential for (1) isotopic fractionation resulting from microbial processing of the plant material; and/or (2) the introduction of ‘new’ carbon through autotrophic fixation during litterbag deployment. Our approach of rinsing detritus prior to analysis, combined with the characteristic decomposition patterns of vascular plants described earlier, suggest the latter is unlikely. Nevertheless, teasing apart the importance of these different processes is difficult. For instance, the increased contribution of alkyl carbon (0–45 ppm) over time in A. marina and both Z. muelleri tissues may be due to the concentration of plant aliphatic carbon (such as the accumulation of leaf waxes we propose above), but may also be due to incorporation of new biomass rich in alkyl C—such as microalgae and/or bacterial biomass (Yao et al. 2019). Such a contribution from algal and/or bacterial sources might also explain the contrasting directional $\delta^{13}C$ shifts in our study. Compound-specific isotope analyses of temperate Australian mudflats have revealed bacterial and algal fatty acids signatures across a range of $\delta^{13}C$ values (-12.3 to -26.4‰), with most sitting between the bulk values we recorded for fresh or decomposed C3 trees and seagrass tissues (Cook et al. 2004). Further evidence for a contribution of heterotrophic biomass to isotope shifts might be seen in the moderate-strong predictive capacity observed for $\delta^{15}N$ was Z. muelleri leaf wrack ($R^2 = 0.69$; prediction RMSE = 0.6‰), which was also the only tissue to show a discernible
shift in $\delta^{15}$N signature over the course of deployment (Fig. 5). Significant peaks and troughs in the $\delta^{15}$N $\beta$-coefficient plot at 68, 76, 105, 174 and 177 ppm (Figure S5) suggest a mix of protein plus non-plant (e.g. chitin) and/or plant carbohydrates may be driving the $\delta^{15}$N shift.

Even though our PLS regression analyses showed that some tissue isotope values can be reliably predicted on the basis of sample composition, the numerous inter- and intra-species complexities we have discussed here preclude a simple explanation of isotopic signature shifts on the basis of plant-material molecular shifts alone. Instead, a combination of compositional shifts in original plant material, isotopic fractionations induced by microbial processing and/or incorporation of new algal or bacterial biomass likely drive changes in isotope signatures during in situ decomposition.

Implications for stable isotope research

1. Significant shifts in both the molecular composition and stable isotope signatures of each of our four study tissues emphasise the need for consideration and accounting of decomposition effects in source-attribution studies. While the selection of end-members will vary with each study’s specific objectives, our findings show that decomposition stage (e.g. live tissue vs litter collected at a specific position along the decay continuum) is a crucial factor in coastal wetland studies. In some circumstances it might be appropriate to apply post-hoc fractionation correction factors (e.g. using relationships like those presented in Fig. 4), though this will depend upon data availability for a given species and/or setting, and the nature and strength of any relationships.

2. An increasingly common use of $\delta^{13}$C and/or $\delta^{15}$N in coastal wetland studies is to demonstrate and/or quantify the stability of carbon down sediment cores (e.g. Adame et al. 2019). Based on the strength of some of our predictive models (Fig. 6), we argue it might be possible to utilise $\delta^{13}$C and/or $\delta^{15}$N as a quantitative indicator of decomposition status, though capacity will vary among species and isotopes. To achieve this, however, further investigation of the relationships between decomposition and isotopic fractionation are required for a more diverse range of species. Investigation is specifically required for belowground tissues, which (i) are poorly represented in fractionation studies; (ii) make significant contributions to long-term blue carbon storage (Donato et al. 2011; Lamont et al. 2019); and (iii) may differ in response to aboveground tissues of the same species (see comparisons of leaf and root/rhizome for Z. muelleri in this study). Further quantification of early versus late stage decomposition effects is also needed to bridge the temporal gap between most decomposition studies (days to a few years) and blue carbon sequestration (decades to millennia).

3. Most studies which incorporate stable isotope approaches assess the isotopic signatures of their target organisms and/or end-members using bulk samples. The bulk sample approach in this study is therefore the appropriate scale for initial investigations as it integrates decomposition and isotopic dynamics over a broad molecular range (i.e. all of the major C functional groups in a tissue). As we describe above, however, there are challenges in determining the relative importance of specific molecules (if any) to isotopic shifts using this bulk approach. In many cases, compound-specific stable isotope analysis and other emerging techniques may be needed to isolate and quantify the specific molecular drivers of isotopic fractionation (Geraldi et al. 2019; Larsen et al. 2013). New insights will emerge from the complimentary use of both bulk sample and compound-specific approaches.

4. We cannot discount the possibility that the disparate deployment conditions and duration influenced the disparate outcomes we observed among the tree species and seagrass. While there were sufficient contrasts within each experimental setup to suggest non-experimental artefacts, the deployment of all tissue types under identical conditions would help to confirm some of the biochemical processes we propose above. There is also a need for controlled experiments to quantify the importance of any microbial fractionation effects and/or new, in-situ autotrophic fixation on litterbag experiments and stable isotope signatures.

Conclusions

Our approach of analysing fresh plant materials and successive bulk litter samples via both stable isotope mass spectrometry and $^{13}$C NMR spectroscopy provided an opportunity to understand the nature of compositional changes during in-situ plant material
diagenesis and its relationship with bulk stable isotope signatures in coastal wetlands. Across all tissues assessed, we observed statistically significant shifts in sample composition across the course of deployment. These shifts can be largely explained by the preferential loss of more labile constituents and concentration of more resistant macromolecules, such as lignin and leaf waxes, though the potential for incorporation of new microbial biomass cannot be discounted.

At the same time, we recorded significant variations in sample $\delta^{13}C$ over the course of deployment for all tissues assessed, and in sample $\delta^{15}N$ for three of the four tissues. While $\delta^{15}N$ variations showed few, if any, clear temporal trends, we observed exponential decays in $\delta^{13}C$ for C. glauca and A. marina, and approximately linear decays for the seagrass tissues. Importantly, these $\delta^{13}C$ shifts were in opposite directions for the two tree species (enrichment in $\delta^{13}C$ over time) compared to the seagrass (depletion in $\delta^{13}C$ over time).

The capacity to predict sample stable isotope values from $^{13}C$ NMR spectra varied among plant taxa, and among the two isotopes tested. While predictive capacity for $\delta^{13}C$ was high for C. glauca and A. marina and moderate for seagrass, inspection of regression $\beta$-coefficients highlighted inter- and intra-species complexities, which preclude a simple explanation of isotopic signature shifts based on plant molecular shifts alone. Instead, we hypothesise that a combination of compositional shifts in original plant material, isotopic fractionations induced by microbial processing and/or incorporation of new algal or bacterial biomass likely drive changes in isotope signatures during in situ decomposition.

Our findings add to a growing body of literature advocating caution in the use and interpretation of stable isotopes as bioindicators of blue carbon provision. We emphasise the need for: (1) accounting of diagenesis effects, and/or using appropriately decomposed litter end-members when attempting source-tracing; and (2) controlled decomposition studies and/or compound-specific analyses to better understand the relationships of between sample composition and isotopic signatures across a range of coastal plant taxa and decomposition settings.

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Author contributions JK conceived the study, undertook data analysis and prepared all figures. JB also undertook data analysis. STT and LC conceived the original litterbag experiments and undertook sample collection. All authors contributed to the generation of data and the writing and reviewing of the manuscript.

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Data availability The datasets generated during and/or analysed during the current study are available in the supplementary material.

Code availability Not applicable.

Declarations

Conflict of interest All Authors declare that they have no competing interest.

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