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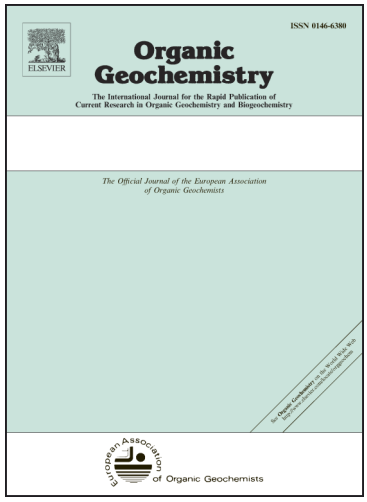

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Investigating the carbon isotope composition and leaf wax *n*-alkane concentration of C_3 and C_4 plants in Stiffkey saltmarsh, Norfolk, UK

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ABSTRACT

The carbon isotope composition of terrestrial plants records valuable ecophysiological and palaeoecological information. However, interspecies variability in $^{13}C/^{12}C$, at both the bulk and compound-specific (CS) level, requires further exploration across a range of ecosystem types. Here, we present bulk and *n*-alkane $\delta^{13}C$ values, and *n*-alkane concentrations, from seven plants (C_3 and C_4) growing in a temperate UK saltmarsh. Inter- and intra-species variation in *n*-alkane $\delta^{13}C$ values among C_3 plants ranged from 8‰ (*n*-C_{31}) to 10‰ (*n*-C_{27}) across the 2011 and 2012 growing seasons, exceeding variability in bulk tissue (7‰). In contrast, the C_4 monocot showed < 2‰ seasonal shifts in bulk and CS values. As a result of the variability in our CS data, we calculate that *n*-alkane based C_3/C_4 reconstructions in temperate saltmarshes have a maximum uncertainty of ~11%. For dicots and succulents, seasonal bulk and CS $\delta^{13}C$ trends diverged, while for C_3 and C_4 monocots, bulk and CS values followed similar temporal patterns. Apparent biosynthetic fractionation varied from –4 to –10‰ for C_3 plants, and reached –13‰ for the C_4 monocot. We explain discrepancies between bulk and *n*-alkane $\delta^{13}C$ values by referring to possible interspecies variation in salinity adaptation, which may influence the partitioning of pyruvate, shifting the isotopic composition of lipid biomarkers. These findings open new avenues for empirical studies to further understand the metabolic processes fractionating carbon during the synthesis of *n*-alkanes, enhancing interpretation of the biomarker signal from the geological record.

Keywords: C_3 vegetation, C_4 vegetation, carbon isotopes, *n*-alkanes, biosynthetic fractionation, saltmarsh

1. Introduction

The $^{13}C/^{12}C$ ratio of plant tissue can be used to distinguish between C_3 and C_4 species (Park and Epstein, 1960; O'Leary 1988; Farquhar et al., 1989) and has been extensively used in the reconstruction of past climates (e.g., Meyers, 1997, 2003; Kohn, 2010; Tipple et al., 2010; Royles et al., 2012). Leaf wax *n*-alkyl $^{13}C/^{12}C$ can be interpreted in a manner similar to that for bulk plant tissue, and therefore has also been used to identify shifts in the relative percentage of C_3 and C_4 vegetation in the sedimentary record (e.g., Huang et al., 2000, 2001; Freeman and Colorusso, 2001; Schefuß et al., 2003; Tipple and Pagani, 2007). However, such reconstructions, whether at the bulk or biomarker level, are subject to uncertainties as a result of variation in carbon isotope composition among different plants.

Meta-analysis of the carbon isotope composition of global C_3 plant biomass has identified significant interspecies variation in $^{13}C/^{12}C$ ratios, with bulk $\delta^{13}C$ values from trees, shrubs and flowering plants ranging from –21 to –35‰ (Diefendorf et al., 2010). This interspecies variation in $\delta^{13}C$ among C_3 plants can complicate the derivation of appropriate end-members in organic matter mixing models for reconstructing C_3 vs C_4 vegetation cover, resulting in large propagated errors of approximately 20% (Castañeda and Schouten, 2011). It is thought highly probable that the variability reported for bulk plant tissue will be reflected in leaf wax biomarker $^{13}C/^{12}C$ composition (Castañeda and Schouten, 2011). Further studies quantifying

interspecies variability in both bulk and *n*-alkane carbon isotope composition from a broad range of biomes are therefore necessary, to constrain the percentage uncertainty associated with reconstructing vegetation cover using the carbon isotope composition of terrestrial plants (Castañeda and Schouten, 2011).

Several recent studies have demonstrated that leaf wax biomarker $\delta^{13}C$ values can vary considerably among certain plant life forms growing at the same location (e.g., Diefendorf et al., 2011, 2015b; Dungait et al., 2011). Diefendorf et al. (2011) found that *n*-alkane $\delta^{13}C$ values for a range of evergreen and deciduous gymnosperm and angiosperm trees reached 10‰, while Dungait et al. (2011) identified interspecies variation of 11‰ in the carbon isotope composition of fatty acids from mixed temperate grasslands. Castañeda et al. (2009) reported ranges of –30 to –39‰ for C_3 trees and shrubs, and –15 to –25‰ for C_4 plants. The fact that different plant species produce widely varying amounts of *n*-alkanes (Tanner et al., 2007; Diefendorf et al., 2011, 2015b) introduces further complexity when interpreting biomarker-based $\delta^{13}C$ reconstructions, as different species may exert a greater or lesser bias over the sedimentary record as a result of the amount of *n*-alkyl lipids they produce. Because of the relatively limited number of empirical observations of interspecies variation in bulk and leaf wax biomarker $\delta^{13}C$ values in extant plants, surveys of vegetation from additional diverse biomes are required to refine interpretation of plant archives in both modern and ancient contexts (Castañeda and Schouten,

2011). The concentration of *n*-alkanes produced by different species also needs to be quantified, to take into account differences in leaf wax production among plants at a single geographical location.

This paper presents bulk and *n*-alkane $\delta^{13}C$ data from seven C_3 and C_4 plants growing at Stiffkey marsh on the north Norfolk coast, UK, collected over a period of 15 months in 2011 and 2012. We also determined the concentration of *n*-C_{27}, *n*-C_{29} and *n*-C_{31} alkanes produced by these plants, as these are the most common homologues used in palaeoclimatic and palaeoenvironmental reconstructions (e.g., Sachse et al., 2012). All of the plant species we analysed are found across the coastlines of continental Europe, and in some cases also have ranges that extend to Africa, Australia and China (Akhani et al., 1997). In addition, saltmarshes are critical components of the global carbon cycle, with ~50% of organic carbon stored in marine sediments found in vegetated costal sedimentary environments (Duarte et al., 2005; 2013). Analysis of the leaf wax biomarker molecular and carbon isotope properties of common saltmarsh plants is therefore valuable for the interpretation of coastal leaf wax *n*-alkyl lipid data from a broad range of vegetated coastal habitats. We further use the new empirical data presented here to evaluate the potential uncertainty associated with C_3 vs C_4 reconstruction in a temperate saltmarsh.

2. Site description

Stiffkey saltmarsh dates from the Holocene and is typical of the open coast back-barrier saltmarshes found along the north Norfolk coastline (Moeller et al., 1996; Allen, 2000) (Fig. 1). This type of marsh has broad sand flats fronting gravel barriers that surround vegetated upper marshland containing fine-grained, silty sediments (Boomer and Horton, 2006).

Like many saltmarshes worldwide, Stiffkey can be divided into different ecological zones/niches primarily based upon topography and the degree of tidal inundation (Davy et al., 2011). The low marsh (LM) is comprised principally of sand flats, and is separated from the upper marsh (UM) by a well-drained gravel/sand ridge (R) formed by onshore emplacement of offshore barrier sediments (Boomer and Woodcock, 1999) (LM, R and UM shown as grey circles, inset map Fig. 1). The LM sand flats are approximately 2.5 m Ordnance Datum (OD), while the UM is 2.8 m OD. The R stands approximately 1 m proud of the surrounding marsh surface. Seawater inundation onto the UM is by tidal flow through a dendritic channel network across the marsh, and also by spring tidal inundation. Neap tides range from 2 to 3 m, although they can be as low as 0.2 m (Pye, 1992; Callaway et al., 1996). Spring tides can be in excess of 5 m, and storm surges from the North Sea can occur (Callaway et al., 1998; Andrews et al., 2000).

Vegetation cover at Stiffkey displays discrete zonation, linked to topography and the extent of tidal inundation (Jeffries, 1977; Davy et al.,

2011). Visual examination of C_3 plant cover during sampling revealed that at the specific sampling locations, the LM is colonised by *Salicornia europaea* and *Limonium vulgare*, and occasionally the woody evergreen shrubs *Atriplex portulacoides* and *Suaeda vera*. The R site supports growth of two monocots *Elytrigia atherica* and *Phragmites australis*, while *S. vera* and *A. portulacoides* also grow in • 1 m high bushes. *L. vulgare, A. portulacoides* and *S. vera* dominate the UM, with *S. europaea* being prevalent around lower lying brackish pools. Only one C_4 species is found at Stiffkey, the monocot *Spartina anglica*, which is typically a pioneer species in the LM sand flats, and also occurs around the brackish low lying pools in the UM.

The selected species at Stiffkey vary in terms of their leaf morphology, ranging from *S. vera* with leaves that are ~2 mm long, to the reed *P. australis* which has large broad-blade leaves that can reach ~30 cm in length. Life strategies are also different, with evergreen species *A portulacoides* and *S. vera* living alongside perennials such as *L. vulgare* and annuals such as *S. europaea*. These plants are also different at the biochemical level: the compatible solutes they use for osmoregulation and amelioration of the harsh saltmarsh conditions (e.g., proteins, amino acids, sugars/carbohydrates), show a degree of species specificity (Bohnert and Jensen, 1996; Ullrich, 2002). These biological mechanisms are important since their existence is not limited to saltmarsh plants – indeed, such adaptations are widely identified in species tolerant to drought, freezing

temperatures, and high UV light (Bohnert and Jensen, 1996; Wanner and Junttila, 1999; Verslues et al., 2006; Jorge et al., 2015). Results from this study are therefore highly relevant to a range of different biomes where plants experience environmental stresses.

3. Methodology

3.1. Sampling strategy

Seven plant species (Table 1) were sampled throughout the 2011 (June, August, October) and 2012 (March, May, August, September) growing seasons at Stiffkey saltmarsh, Norfolk, UK from the three sampling sites (grey circles) shown in Fig. 1. Three replicate samples of each species were collected at each sampling interval. Each of these replicates comprised a minimum of five leaves (depending on leaf morphology), sampled from at least three individual plants to ensure a representative signal was obtained from each species. For the reed *P. australis,* ~5 leaves were collected, while for the leaf succulent *S. vera* collection exceeded 100 leaves. Care was taken to always select whole, undamaged, non-senescent leaves. During sampling, the most recent growth was selected from all plants. For the two evergreen species *A. portulacoides* and *S. vera,* sampling therefore focused on the leaves at the tips of stems, to ensure that new season growth was selected for study. All leaves sampled were, as far as possible, subject to similar

levels of exposure to wind, rain and sunlight in order to avoid bias on the basis of canopy position and/or particularly sheltered locations within marsh sub-environments. All plant samples were placed in paper envelopes, and dried at 40 °C for ~48 h upon returning to the laboratory, and stored in a dry, dark environment until required for further analysis.

3.2. Bulk $\delta^{13}C$ analysis

Plant leaf samples were ground into a fine powder using a cryogenic mill (SPEX Freezer/Mill), weighed into tin capsules, crimped, and then analysed for $\delta^{13}C$ using a Delta XP ThermoFisher isotope ratio mass spectrometer interfaced with a CosTech elemental analyser. An in-house standard, casein ($\delta^{13}C = -23.37‰$, previously calibrated against the International Atomic Energy Agency (IAEA) reference materials during an inter-laboratory comparison exercise as part of EU Project SMT4-CT98-2236), was used to correct for drift in raw analytical data. Further correction for scale compression was then carried out using both the casein standard and an in-house collagen standard, also calibrated against IAEA references materials during the EU Project SMT4-CT98-2236. Reproducibility of the CO_2 reference gas $\delta^{13}C$ measurements was ± 0.03‰, while repeated $\delta^{13}C$ measurements of the in-house collagen standard varied by ± 0.3‰ ($n = 28$). All plant samples were analysed in duplicate; absolute differences between analytical replicates of the same sample for $\delta^{13}C$ did not exceed 0.6‰.

3.3. n-Alkane analysis

Leaf wax *n*-alkanes were extracted from whole leaves by sonication with HPLC grade hexane to obtain the total lipid fraction, as described in detail in Eley et al. (2012, 2014). Briefly, analysis of the molecular distribution and concentration of *n*-alkanes for each species was carried out by injection into an Agilent 7820A gas chromatograph equipped with a flame ionisation detector and a DB-5 capillary column (30 m × 0.32 mm × 0.25 µm) (Agilent Technologies Inc., Santa Clara, USA). *n*-Alkanes were identified by comparison of their elution times with an *n*-C_{16} to *n*-C_{30} alkane standard (A. Schimmelmann, Indiana University). Quantification of leaf wax *n*-alkanes was carried out by the addition of a known concentration of a C_{15} *n*-alkane standard (Sigma Aldrich) to each sample prior to analysis.

The carbon isotope composition of *n*-alkanes was determined using a Delta V Advantage ThermoFisher isotope-ratio mass spectrometer interfaced with GC-Isolink Trace GC combustion and high temperature conversion systems, using the same type of GC capillary column (Agilent DB-5) as described above for the GC-FID analysis. $\delta^{13}C$ values of *n*-alkanes are reported based on duplicate analyses of well-resolved peaks and expressed relative to Vienna Pee Dee Belemnite (VPDB) based on in-house reference gases (CO_2, BOC) adjusted daily using a standard mixture of *n*-C_{16} to *n*-C_{30} alkanes (A. Schimmelmann, Biogeochemical Laboratories, Indiana

University). Reproducibility of reference gas peak CO_2 $\delta^{13}C$ values (analysed at the beginning and end of each sample measurement) did not exceed ± 0.05‰. Differences between repeat measurements of the same analytical sample did not exceed 0.5‰. Root mean square (RMS) errors for standard measurements (*n*-C_{16} to *n*-C_{30} alkanes) did not exceed 0.5‰ ($n = 144$) throughout sample analysis. To assess the heterogeneity of the carbon isotope composition of a single plant species, we analysed two of the sample replicates we collected for each plant, and calculated the absolute difference in $\delta^{13}C$ values between these measurements. For all species, this variability did not exceed 2‰.

4. Results and discussion

Previous studies have measured interspecies variability in bulk and leaf wax *n*-alkane $^{13}C/^{12}C$ ratios to a limited extent, resulting in uncertainty when deriving appropriate end-members for use in source apportionment models seeking to reconstruct the proportions of C_3 and C_4 vegetation contributing to an ecosystem (Castañeda and Schouten, 2011). In addition, while variability in *n*-alkane production has been documented among a relatively small number of plant species and functional types (Tanner et al., 2007; Diefendorf et al., 2011, 2015b), this parameter remains under-explored across a range of different biomes. In this study, we provide new bulk and *n*-alkane $\delta^{13}C$ data from six C_3 plants and one C_4 plant growing in a

temperate UK saltmarsh. We evaluate several mechanisms that may account for inter- and intra-species variability in carbon isotope composition at the bulk and CS level, and consider the implications of variation in the relationship between bulk and *n*-alkane $\delta^{13}C$ values for palaeoenvironmental reconstruction. We also investigate the extent of interspecies variation in *n*-alkane production among these species, as this factor is important in determining the influence that each species will have on the concentration-weighted carbon isotope composition ultimately recorded by the *n*-alkanes entrained in the saltmarsh sediments (Kirkels et al., 2013).

4.1. Bulk $^{13}C/^{12}C$ composition

Bulk tissue from the six C_3 saltmarsh species sampled across the 2011 and 2012 growing seasons showed maximum variability in the monocot grass *E. atherica* (~ 5‰; Fig. 2, Tables 2 and 3). In contrast, the species with the most consistent bulk $^{13}C/^{12}C$ composition throughout the entire sampling period was the reed *P. australis,* with a variability of only 0.9‰. The seasonal shift in $\delta^{13}C$ values was greater in the two evergreen species *A. portulacoides* and *S. vera* (3‰), compared with that observed in the perennial *L. vulgare* and the annual succulent *S. europaea* (2‰). The C_4 monocot *S. anglica* had a bulk isotope signal that varied by less than 1‰ in both 2011 and 2012 (Fig. 2).

For the C_3 species, there was no direct relationship between seasonal patterns in ^{13}C-enrichment or depletion and plant life form (e.g., monocot, flowering shrub, succulent) or growth strategy (e.g., perennial, annual, evergreen) (Fig. 3). Indeed, among the sampled species the most ^{13}C-enriched bulk tissue was found in the evergreen shrub *A. portulacoides*, while the most ^{13}C-depleted tissue was found in the evergreen succulent *S. vera*. ^{13}C-enrichment in bulk tissue was generally greater in the summer months, and lower in the early growth season, e.g., March and May 2012. Total interspecies variation in bulk carbon isotope composition was 6.5‰, ranging from –31.4 to –24.9‰ (Figs. 2 and 3, Table 2 and 3).

The observed variability in the bulk $\delta^{13}C$ values of the C_3 species at Stiffkey could potentially be explained by changes in plant physiology and biochemistry in response to environmental stresses over the growth season. Saltmarsh plants are subject to a range of adverse conditions, including salinity, nitrogen limitation, root anoxia, waterlogging, changes in redox potential, and high concentrations of toxic sulphur-containing chemicals (Drake, 1989; Parida and Das, 2005; Maricle et al., 2007; Song et al., 2009; Davy et al., 2011). The periodic nature of these stress factors drive a suite of adaptive responses in saltmarsh plants, such as seasonal changes in leaf physiology/morphology and shifts in biochemistry (e.g., Drake, 1989; Parida and Das, 2005; Maricle et al., 2007). These adaptations therefore have the potential to influence the bulk chemistry of leaf tissue and leaf wax *n*-alkane carbon isotope values. Although in-situ measurements of parameters

such as salinity, soil pH, and soil water potential were not carried out as part of this study, the environmental conditions of Stiffkey marsh have been previously documented. The site was the subject of a series of studies in the 1970's, and seasonal shifts in water availability, nutrient stress and salinity are therefore well known (Jefferies, 1977; Jefferies and Perkins, 1977). As these edaphic parameters have been shown to influence the bulk $\delta^{13}C$ composition of plant material via their impact on C/C_a (e.g., Arens et al., 2000 and references therein), we have interpreted interspecies and seasonal variation in $^{13}C/^{12}C$ at the bulk level as a physiological response of these plants to changes in environmental conditions (Dungait et al., 2008, 2010; Cernusak et al., 2013).

4.1.1. The role of water availability

LM environments at Stiffkey (Fig. 1) are subject to tidal inundation throughout the year, however, due to marsh topography summer tides do not always cover the UM (Jefferies, 1977). As a result of evapotranspiration and relatively low rainfall in summer months, the UM, and other elevated environments at Stiffkey such as the Ridge, have drier soils with lower water availability, relative to those of the LM (Jefferies, 1977). A decrease in soil moisture availability gives rise to concurrent reductions in photosynthesis, transpiration and stomatal conductance (Farquhar et al., 1989; Hubbard et al., 2001). The reduction in stomatal conductance results

in diminishing C_i values, and increases in the $\delta^{13}C$ value of bulk plant material (Farquhar et al., 1989).

Positive shifts in plant bulk $\delta^{13}C$ during a growing season have previously been interpreted as evidence of plants adapting their water use strategies to enhance water use efficiency (WUE) (e.g., Farquhar and Richards, 1984; Donovan and Ehleringer, 1992; Ehleringer et al., 1993; Siebt et al., 2008). At Stiffkey, bulk $\delta^{13}C$ data for the monocot *E. atherica* (Ridge) show a positive shift of 2 to 3‰ as the summer progresses in both 2011 and 2012 (Fig. 3). Similar shifts are seen in *A. portulacoides* and *S. vera* at the Ridge (Fig. 3), suggesting that this sub-habitat might experience a degree of water stress in late summer, when rainfall decreases and soil evaporation rises with the warm temperatures. However, not all plants at the Ridge responded in the same way across the 2011 and 2012 growing seasons. The reed *P. australis*, in particular, shows very little seasonal variation in bulk $\delta^{13}C$ composition across both sampling years (Fig. 3). *P. australis* has substantial root networks, reaching depths of up to 1.5 m, and an extensive perennial rhizome, which allows it to make use of water from a wide area around the stand (Lissner and Schierup, 1997). Therefore, the remarkably consistent $\delta^{13}C$ signature of *P. australis* may simply reflect the fact that this particular species is less susceptible to the effects of seasonal changes in water availability than other plants at the Ridge.

4.1.2. The role of salinity stress

Osmotic stress due to increasing salinity has also been shown to result in a positive shift in plant $\delta^{13}C$ values of between 3 and 10‰ (Arens et al., 2000; Wei et al., 2008; Ladd and Sachs, 2013). In non-halophyte species, metabolic effects of increasing salinity include stomatal closure, which gives rise to a positive shift in $\delta^{13}C$ as a result of a decrease in C_i(Guy et al., 1986; Farquhar et al., 1989; Arens et al., 2000). Studies of halophytes also show a positive shift in bulk tissue $\delta^{13}C$ when grown in saline soils, as an increase in salinity generally requires even salt-tolerant plants to enhance their WUE, resulting in ^{13}C-enriched biomass (Guy et al., 1986). Although not regularly inundated by tides, increasing salinity at the ridge may result from an upward movement of brackish water ascending through soil capillaries as evaporation from the surface soil layers increases during warmer months (Rowell, 1994). Given that salinity impacts upon C_i/C_a in a similar fashion to water stress, it is possible that the seasonal trends observed in bulk $\delta^{13}C$ values of *E. atherica*, and indeed of *A. portulacoides* and *S. vera* (Fig. 3) arise from a change in gas exchange rates driven by a combination of water and salinity stress.

4.1.3. Juvenile vs adult plants

The bulk $\delta^{13}C$ values of the stem succulent *S. europaea,* which grows in the LM, become higher during the summer months (Fig. 3). As the LM

receives regular tidal inundation throughout the year, it is unlikely that water stress alone is responsible for these shifts. Equally, as the low marsh is consistently inundated by tides, the conditions for hypersalinity rarely occur and therefore changes in salinity are unlikely to explain all of the seasonal shifts in bulk $\delta^{13}C$ values observed in this stem succulent. Root anoxia, another environmental mechanism driving positive shifts in plant $\delta^{13}C$ (Arens et al., 2000) is also likely to be constant across the year in the tidal, water-logged sand flats. This suggests that a temporal factor, such as the maturation from juvenile plants into reproductive adults (Arens et al., 2000), may be responsible for the positive shifts in the $\delta^{13}C$ values of *S. europaea* toward the end of the growing season.

As plants grow from juveniles to adults, rooting depths and water potentials are known to increase (Donovan and Ehleringer, 1991). Juvenile semi-arid shrubs have previously been shown to be less WUE than adult plants (Donovan and Ehleringer, 1994), and have higher rates of photosynthesis, stomatal conductance and transpiration than reproductive adults (Donovan and Ehleringer, 1992). A gradual increase in WUE as these plants mature, for example resulting from greater efficiency in maintaining transpiration rates, may account for the positive shift in late summer observed for *Salicornia* (Fig. 3). Alternatively, as the carbon isotope composition of bulk plant tissue is comprised of a mixture of different compounds (each with distinctive isotope values; Schmidt, 2003), seasonal shifts in plant biochemistry or the allocation of carbon to other organs may

also account for the observed trends in this annual stem succulent.

4.2. n-Alkane $^{13}C/^{12}C$ composition

Carbon isotope measurements of *n*-C_{27}, *n*-C_{29} and *n*-C_{31} are presented in Fig. 3 and Tables 2 and 3. Maximum seasonal variation in the different C_3 species sampled was 10.2‰ for *n*-C_{27}, 8.6‰ for *n*-C_{29}, and 8.4‰ for *n*-C_{31} (Fig. 3). In contrast for the C_4 monocot, the maximum variability of *n*-C_{27} was 1.9‰; *n*-C_{29} and *n*-C_{31} both shifted by only 1.7‰ across the whole 2011 and 2012 sampling period (Fig. 3). Using the concentration of these three *n*-alkane homologues (Tables 4 and 5; Section 4.3 below) we also calculated the concentration-weighted average (WA) *n*-alkane carbon isotope composition of each plant species for each sampling interval (Tables 2 and 3) using Equation 1, where $[C_{27}]$, $[C_{29}]$ and $[C_{31}]$, represent the concentration of *n*-C_{27}, *n*-C_{29} and *n*-C_{31} alkanes produced by each species:

$$\delta^{13}C_{WA} = (([C_{27}] * \delta^{13}C_{C27}) + ([C_{29}] * \delta^{13}C_{C29}) + ([C_{31}] * \delta^{13}C_{C31})) / ([C_{27}] + [C_{29}] + [C_{31}])$$

Eq. 1

and found that the maximum seasonal variation in this value was 9‰, again exceeding the range observed for bulk tissue.

For the C_3 species (but not the C_4 monocot), intra-species seasonal variability was also different from bulk tissue, with the greatest variability in lipid $\delta^{13}C$ values (5‰) during the sampling period observed in *L. vulgare*

(Fig. 3). *P. australis* and *S. europaea* in contrast had the most consistent *n*-alkane $\delta^{13}C$ profiles throughout 2011 and 2012 (Fig. 3). The most positive *n*-alkane $\delta^{13}C$ values were consistently found in *A. portulacoides* (as opposed to *P. australis* at the bulk level), while the most negative values were recorded for *E. atherica* and *S. vera*. Overall, seasonal variation in the carbon isotope composition of *n*-C_{27}, *n*-C_{29} and *n*-C_{31} alkanes from C_3 plants was 2–4‰ higher than that observed at the bulk level, while the C_4 monocot displayed a similar degree of variability (up to 2‰) in bulk and *n*-alkane carbon isotope values (Tables 2 and 3).

Despite the fact that mechanisms driving bulk carbon isotope composition are likely to influence the carbon isotope composition of *n*-alkanes, leaf wax *n*-alkane $\delta^{13}C$ values from the Stiffkey plants do not consistently follow the same seasonal patterns as observed in bulk tissue (Fig. 3). This therefore suggests that additional mechanisms may be responsible for influencing individual *n*-alkane $\delta^{13}C$values. Fig. 4 highlights that there was considerable intra- and inter-species variability in the relationship between bulk and weighted average *n*-alkane carbon isotope ratios among the C_3 species, with *n*-alkane carbon isotope values ranging from 4 to 13‰ lower than bulk values across the 2011 and 2012 growth seasons.

Among the sampled plants, WA $\delta^{13}C$ values from *E. atherica* were generally the most ^{13}C-depleted relative to bulk tissue resulting in the greatest fractionation ($\varepsilon_{wa/bulk}$), while WA values from *S. europaea* and *A.*

portulacoides were consistently the most ^{13}C-enriched, resulting in these species having lower values of $\varepsilon_{wa/bulk}$ between *n*-alkane and bulk $\delta^{13}C$. *S. vera*, *L. vulgare* and *S. europaea* recorded the maximum seasonal variability (4‰) in the relationship between their WA and bulk tissue $\delta^{13}C$ values, while *P. australis* (1‰), *E. atherica* (2‰) and *A. portulacoides* (2‰) were the most consistent. Seasonally, the greatest ^{13}C-depletion in WA values versus bulk tissue generally occurs later in the growth season (e.g., October 2011, August and September 2012), while the least depletion is observed earlier, particularly during June 2011, and March/May 2012 (Fig. 4). A notable exception to this rule is the sample of *L. vulgare* from March 2012, when newly emergent leaves were sampled. The C_4 monocot *S. anglica* had *n*-alkane $\delta^{13}C$ values that were 10–13‰ more negative than bulk tissue.

4.2.1. Mechanisms that may influence the carbon isotope composition of n-alkanes

In order to fully interpret the information contained in the carbon isotope signal of leaf wax *n*-alkanes, it is important to consider the post-photosynthetic processes that may influence their isotope composition. Studies of enzyme-moderated stable isotope fractionation in biological systems have shown that the composition of specific compounds can be strongly influenced by the $^{13}C/^{12}C$ of their precursor carbon source, and any subsequent biochemical kinetic isotope fractionation caused by carbon fluxes

through reaction networks (Hayes, 2001; Hobbie and Werner, 2004; Dungait et al., 2008; Zhou et al., 2015). Tcherkez et al. (2011) proposed that the fluxes of carbon through plant metabolic processes can give rise to considerable redistribution of ^{12}C and ^{13}C that occurs after carbon is fixed during photosynthesis. Processes such as dark respiration, the transport of carbon to sink tissues within the plant, and photorespiration all may influence the carbon isotopic composition of pyruvate, the precursor for the acetogenic pathway producing leaf wax biomarkers (Zhou et al., 2015).

Negative seasonal shifts in the carbon isotope composition of plant components, such as leaves and stems, has been previously shown to relate to the use of different stored reserves within long-lived plants such as trees (e.g., Damesin and Lelarge, 2003). Early in the growth season, for example, buds from *Fagus sylvatica* have been shown to have similar carbon isotope values as starch present in the stem at the end of the growing season, indicating that the early season growth utilised reserves stored from the end of the previous season (Damesin and Lelarge, 2003). Indeed, seasonal shifts in the concentration of starch and soluble sugars in stems have also been observed in beech (Barbaroux and Breda, 2002) and woody plants (Witt and Sauter, 1994). This is significant, as starch is an important source of carbon for the lipid precursor pyruvate (Zhou et al., 2015).

Diefendorf et al. (2011) propose that changes in the flux of pyruvate within a plant cell can shift the isotopic composition of acetogenic lipids such as *n*-alkanes, with ^{13}C-depletion anticipated when lipid production

decreases. The diurnal transport of relatively ^{13}C-enriched sugars from leaves to other plant organs has previously suggested to give rise to a ^{13}C-depleted carbon pool in leaves, resulting in their ^{13}C-depletion in comparison with other plant organs such as roots (Badeck et al., 2005; Gessler et al., 2008). Throughout a growing season, therefore, increases in the demand for sugars from other plant organs (which could arise, for example, during the plant reproductive phase or the accumulation of seasonal reserves; Chapin et al., 1990) may lead to increasingly ^{13}C-depleted carbon in leaves for pyruvate production, and ultimately leaf wax lipid synthesis via the acetogenic pathway. This may account for the observed general seasonal trend among the Stiffkey plants towards more ^{13}C-depeleted *n*-alkane values as the summer progresses (Fig. 3).

While changes in resource use may account for a gross seasonal shift towards ^{13}C-depleted *n*-alkane values, accounting for interspecies differences in *n*-alkane δ^{13}C values and the offset between bulk and *n*-alkane carbon isotope composition, however, requires further consideration. Carbon allocation is known to influence the carbon isotope composition of a range of plant lipids, with effects observed both seasonally and between different plants (Park and Epstein, 1961; Chikaraishi et al., 2004). Indeed, differences between monocots and other woody species in terms of the relative amounts of carbon in ^{13}C-enriched sugars and ^{13}C-depleted compounds such as lignin and lipids have been proposed to account for the greater difference in the δ^{13}C values of lipids relative to bulk tissue in

monocots (Hobbie and Werner, 2004). The relationship between lipid concentration and offset among the Stiffkey plants is not straightforward, however. Fig. 5 shows that the monocot *E. atherica* in particular often has the greatest offset between bulk and *n*-alkane carbon isotope values, despite frequently having relatively high concentrations of *n*-C_{29}. In contrast, *S. europaea* produces the lowest concentrations of *n*-C_{29}, but also has the lowest offset between bulk and *n*-C_{29} carbon isotope values (Fig. 5).

Previous studies of CAM species suggest that shifts in carbon allocation among plants to mitigate water availability has the potential to explain variability of 0.5 to 12.8‰ in the depletion of lipids relative to the carbon isotope composition of bulk tissue (Boom et al., 2014). Although we did not analyse CAM species as part of this study, in principle similar mechanisms could operate in our C_3 and C_4 saltmarsh plants. Changes in carbon allocation among the Stiffkey plants in response to water stress could therefore represent an additional mechanism to account for interspecies differences in *n*-alkane $\delta^{13}C$, observed both among different species, and in one species across a growing season. Such mechanisms could also play a role in driving the differences we observe between bulk and *n*-alkane $\delta^{13}C$ values.

Many plants subject to environmental stresses, including those found at Stiffkey, produce a suite of protective compounds to ameliorate the effects of conditions such as hypersalinity (e.g., Briens and Larher, 1982). These typically include quaternary ammonium compounds, amino acids and

carbohydrates (Briens and Larher, 1982; Hare and Cress, 1997; Rhodes et al., 2002; Ashraf and Foodad, 2007). The production of carbohydrates, in particular, in response to environmental stresses could alter the total concentration of pyruvate within the plant leaves (Rhodes et al., 1986; Good and Zaplachinski, 1994), while enhanced amino acid synthesis could result in shifts in the allocation of pyruvate to different metabolic pathways, factors which have been previously suggested to influence leaf wax biomarker $^{13}C/^{12}C$ values (e.g., Diefendorf et al., 2011). Plant species can be broadly divided into: (a) species that only synthesise high levels of carbohydrates; (b) species that synthesise carbohydrates and nitrogenous compounds; and (c) species that accumulate higher concentrations of nitrogenous compounds relative to carbohydrate compounds (Briens and Larher, 1982). It has been shown previously that some monocot species found at Stiffkey typically produce more carbohydrates, while dicots and succulents preferentially accumulate nitrogenous compounds (Briens and Larher, 1982).

Carbohydrates and amino-acids/nitrogenous compounds typically have different carbon isotope ratios, with amino acids depleted in ^{13}C relative to carbohydrates (Schmidt et al., 2003). Since it is possible that each of these species produce a specific suite of osmoregulatory compounds (e.g., Ullrich, 2002), this could lead to different carbon flow patterns – and thus differences in carbon isotope fractionation – through the acetogenic lipid biosynthesis pathway (Hayes, 2001; Diefendorf et al., 2011) among these

plants. This could explain not only interspecies variation in $^{13}C/^{12}C$ at one sampling interval, but also the reason why this variability shifts across a growing season in response to increasing salinity and decreasing water availability. Future studies of extant plants could explore this in more detail through a combined analysis of the concentration and carbon isotope composition of these compounds, to investigate the nature of their influence on leaf bulk and wax biomarker carbon isotope composition. In addition, in modern saltmarshes, and indeed other biomes, the $\delta^{13}C$ of fixed carbon in plant bulk tissues will be a factor of the $\delta^{13}C$ of atmospheric CO_2, and the species-specific stomatal and mesophyll responses, to ensure a balance is maintained between CO_2 uptake and water loss (McCarroll and Loader, 2004; Flexas et al., 2008; Siebt et al., 2008; Tholen et al., 2012; Werner et al., 2012). Future research could also explore the extent of inter- and intra-species variability at the bulk and molecular level in $\delta^{13}C$ values across a range of temperature regimes, to evaluate how temperature influences the extent of variability observed.

4.2.2. Implications for palaeoecology

The divergent seasonal trends in $\delta^{13}C$ values at the bulk and biomarker levels observed for some plant species illustrate the complexity in calculating an appropriate fractionation factor between bulk and *n*-alkane carbon isotope values. This has important implications for studies seeking to

use leaf wax biomarker information in palaeoecological studies. Firstly, recent research has used bulk and *n*-alkane $\delta^{13}C$ values interchangeably to calculate carbon isotope fractionation ($\Delta^{13}C$) (e.g., Smith et al., 2007; Diefendorf et al., 2010). These studies use apparent fractionation factors ("*εf*") to back-calculate the carbon isotope composition of bulk leaf material from *n*-alkane data, prior to calculating $\Delta^{13}C$ values (e.g., Smith et al., 2007; Diefendorf et al., 2010; 2015a). Some palaeoenvironmental reconstructions have used a fixed value of –4.9‰ to describe this apparent biosynthetic fractionation (Smith et al., 2007; Diefendorf et al., 2010), while more recent publications have used values of –4.6‰ for *n*-C_{29} and –5.0‰ for *n*-C_{31} for conifers, based on studies of extant plants (Diefendorf et al., 2015a,b). Alternative approaches propose that apparent fractionation is affected by environmental conditions and the specific biochemistry of individual species, but is consistently within the range of –5 to –7‰ for C_3 species and –8 to –10‰ for C_4 species (Tipple and Pagani, 2007). Our results show, however, that the current range of fractionation factors used in palaeoenvironmental reconstructions may not always capture the complexity of the relationship between bulk and *n*-alkane carbon isotope values. For example, our C_3 species have *εf* values ranging from –4 to –10‰, while our C_4 monocot has *εf* values of –10 to –13‰ (Tables 2 and 3; Fig. 4). In addition, as a result of the divergent seasonal trends in bulk and *n*-alkane carbon isotope values for some C_3 species at Stiffkey, seasonal patterns in $\Delta^{13}C$ values calculated for the Stiffkey plants differ depending upon whether bulk or *n*-alkane values

are used (Fig. 6). We suggest that calculation of εf values, particularly when it is done interchangeably using either bulk biomass or compound-specific $\delta^{13}C$, requires species-specific information about the magnitudes of the difference between the bulk and biomarker $\delta^{13}C$ values.

Secondly, the seasonal $\delta^{13}C$ trends reported for the Stiffkey plants are significant when considering the fidelity of saltmarsh plant leaf wax biomarkers as proxies for environmental conditions such as salinity. In their study of mangroves, Ladd and Sachs (2013) identified a positive trend between water salinity and $\delta^{13}C$ of *n*-C_{31}, *n*-C_{33} and bulk leaf tissue along a river estuary transect. They interpreted this as a shift in the WUE of mangrove leaves in response to a change in salinity. Our study, however, finds no clear trend for *n*-C_{27}, *n*-C_{29} or concentration-weighted average *n*-alkane $\delta^{13}C$ values to become ^{13}C-enriched in the summer months when hypersalinity is highest. Indeed, across a range of species, including *L. vulgare* (which grows in the UM where hypersalinity would be most expected), *S. europaea, S. vera* and *A. portulacoides*, *n*-alkanes became more ^{13}C-depleted in the months where hypersalinity is common. This suggests that the patterns observed in mangrove *n*-alkane $\delta^{13}C$ values in response to changes in salinity may not necessarily be applicable to a wider range of saltmarsh plants in temperate climates.

4.3. n-Alkane concentrations

Total *n*-alkane concentrations were generally highest in the perennial C_3 herb *L. vulgare* (Figs. 7 and 8; Table 4 and 5), however, consideration of individual chain lengths showed that in March 2012 the C_3 grass *E. atherica* had the highest concentrations of *n*-C_{27} and *n*-C_{29}, but not *n*-C_{31} (Figs. 7 and 8). The lowest concentration of *n*-alkanes in any species sampled was consistently found in *S. europaea*, the C_3 stem succulent. *E. atherica* and *S. anglica* had the highest concentrations of *n*-C_{29} among the monocot species sampled, while the reed *P. australis* generally had less than half the concentration of *n*-C_{29}, although it tended to have ~10 μg/g dry wt more *n*-C_{27}.

When comparing the concentration of *n*-alkanes in the Stiffkey species with previously published data from other plants from different locations, *S. anglica* has similar amounts of each homologue relative to *Spartina alterniflora* sampled from Massachusetts (Wang et al., 2013), but far lower concentrations than those reported for *Spartina patens* sampled in Maine (Tanner et al., 2007). Similar differences are observed when comparing concentrations from other species from the same genus. *Limonium nashii* in Maine (Tanner et al., 2007) had up to 213 μg/g dry wt less *n*-C_{29} than *L. vulgare* from Stiffkey, for example, for the same sampling month. Likewise, when *S. europaea* data from Stiffkey are compared with *n*-alkane concentrations published for *Salicornia depressa* (Tanner et al., 2007; 2010). *S. depressa* has over 80 times higher concentrations of *n*-C_{23} and *n*-C_{25} than *S. europaea*. The variation in *n*-alkane concentrations

observed among the compared species could be due to several mechanisms. Firstly, the degree of wax abrasion due to environmental conditions such as wind ablation, rainfall and tidal inundation could vary between the Stiffkey site and the Machiasport marsh in northeastern Maine investigated by Tanner et al. (2007, 2010). Mechanical stresses such as these can ablate wax crystals from leaves (Shepherd and Griffiths, 2006), and could theoretically give rise to lower overall wax concentrations in exposed sites when compared to sheltered ones. Although plants can replenish their wax layers, this is not always fast enough to maintain typical wax amounts for a particular species (Shepherd and Griffiths, 2006). It is therefore conceivable that plants growing at sites such as Stiffkey, where they are continually exposed to onshore winds from the North Sea, may display lower concentrations of *n*-alkanes than plants of the same species growing in more sheltered conditions. Tanner et al. (2007, 2010) noted that the estuarine site they studied was protected by high cliffs, in a direct contrast to the exposed conditions at Stiffkey. This explanation, however, does not account for the significantly higher concentrations of *n*-alkanes in *L. vulgare* (Stiffkey) compared with *Limonium nashii* (Tanner et al., 2007). Previous studies have reported that within a plant family, different genera can vary widely in respect of *n*-alkane concentrations (Diefendorf et al., 2015b). We therefore surmise that similar genetic variation could account for the differences observed between these *Limonium* species. Alternatively, differences in the growth rates and life strategies of these two species could influence the

relative variation in concentration observed, as the maturity of leaves can also impact upon *n*-alkane concentrations, with more mature leaves having higher *n*-alkane concentrations (Jetter and Shaeffer, 2001). These results demonstrate that site characteristics should be carefully checked when comparing data from similar species growing in different geographical locations, to ensure that such comparisons are appropriate.

Temporal variation in *n*-alkane concentration appears to be species-specific among the Stiffkey plants. The highest concentrations of *n*-C_{27}, *n*-C_{29} and *n*-C_{31} in *A. portulacoides, E. atherica* and *S. vera* were found in March 2012. In *E. atherica* and *A. portulacoides,* concentrations decreased as the growing season progressed – a trend that was particularly noticeable in 2012 (Figs. 7 and 8, Table 5). However, in *S. europaea, S. anglica* and *P. australis n*-alkane concentrations increased at the end of the growing season (Figs. 7 and 8). No clear systematic variation in *n*-alkane concentration was therefore observed with seasonality, suggesting that these trends were not simply a plant response to local environmental conditions. In some species, such as *P. australis* and *S. anglica,* clear differences were observed when leaf wax concentrations from 2011 were compared with those from 2012. *L. vulgare* also had considerable annual variation, with very high concentrations of *n*-alkanes in June 2011, which were not repeated throughout 2012. Other species showed greater consistency across the 2011 and 2012 growing seasons. *E. atherica, S. vera* and *A. portulacoides* in

particular recorded similar profiles in both 2011 and 2012, with concentrations peaking in March 2012 (Figs. 7 and 8).

The concentration of *n*-alkanes in leaf waxes during the growing season can be: (a) increased as a result of plants continually biosynthesizing new organic compounds; (b) decreased due to the wax being abraded away by mechanical stress caused by edaphic factors such as wind and rain; or (c) influenced by environmental/climatic factors such as changes in water availability and the amount of UV light (Shepherd and Griffiths, 2006). As discussed previously, Stiffkey saltmarsh is situated on the north Norfolk coast, and topographically does not offer plants much in the way of protection from onshore winds and rainfall. Differences in local weather conditions, altering the amount of wax ablation, are therefore potential explanations for the differences in alkane concentration observed between 2011 and 2012.

4.4. Evaluating uncertainty in C_3/C_4 reconstructions in a temperate saltmarsh

Using the carbon isotope data from the C_3 and C_4 plants, we calculated the percentage uncertainty associated with using each of the homologues to reconstruct C_3/C_4 vegetation cover following the approach of Castañeda et al. (2009). For the C_3 plants, uncertainties (based on the standard deviation of measured values for all species across the 2011 and

2012 sampling periods) were 8% for *n*-C_{27}, 7‰ for *n*-C_{29} and 6% for *n*-C_{31}. For the C_4 monocot *S. anglica*, the relative invariance in carbon isotope values at the bulk and molecular level meant that percentage uncertainties were far lower, all no more than 3%. As a result, when reconstructing C_3 vs C_4 plant cover at Stiffkey, the maximum percentage uncertainty is likely to be *c.* 11% using *n*-C_{27}, 10% using *n*-C_{29} or 9% using *n*-C_{31}. If using a concentration weighted average carbon isotope value for each plant species (*n*-C_{27}, *n*-C_{29} and *n*-C_{31}), then the percentage uncertainty is 9%.

This level of uncertainty is far lower than that reported for reconstructions of Sahara/Sahel C_3/C_4 dynamics from North Africa (20%, Castañeda et al., 2009), although here average *n*-alkane $\delta^{13}C$ values and percentage uncertainties were calculated based on previously published leaf wax biomarker data from Africa, South America and Australia (Castañeda et al., 2009). Castañeda and Schouten (2011) hypothesise that the wide range of bulk leaf $\delta^{13}C$ values published by Diefendorf et al. (2010) indicate that such percentage uncertainties may even be higher than 20% when using *n*-alkane measurements as opposed to bulk. Our results show, however, that while it is true that interspecies variation is higher among *n*-alkane carbon isotope values than bulk tissue, constraining uncertainty for our geographical location results in a maximum percentage uncertainty of 11% if a single alkane homologue is used, or 9% if a concentration-weighted average $\delta^{13}C$ value is used. This suggests that future investigations of C_3 and C_4 vegetation dynamics may be improved through quantifying percentage

uncertainty for a specific site, biome type or depositional environment, rather than relying on averages of global or multi-regional $\delta^{13}C$ values.

5. Conclusions

In this contribution, we add to limited actualistic leaf wax concentration and compound-specific (CS) $\delta^{13}C$ data, reporting measurements from C_3 and C_4 plant species common to temperate saltmarshes. *n*-Alkane concentrations among the studied species vary by almost an order of magnitude, with the perennial dicot *L. vulgare* producing the highest concentration of *n*-C_{27}, *n*-C_{29}, and *n*-C_{31} and the stem succulent *S. europaea* the lowest. We find that interspecies variability in individual *n*-alkane $\delta^{13}C$ values is up to ~4‰ higher than in bulk tissue: from 10‰ for *n*-C_{27}, 9‰ for *n*-C_{29} and 8‰ for *n*-C_{31}. Further, among the C_3 dicots and succulents, seasonal trends in bulk and CS $\delta^{13}C$ values differ, resulting in variation in the offset between bulk and *n*-alkane carbon isotope composition of –4 to –13‰. For C_3 and C_4 monocots, however, seasonal trends in carbon isotope values are similar at the bulk and CS level. As a result of these divergent trends, we conclude that it may not be valid to use bulk and *n*-alkane $\delta^{13}C$ data for all plant life forms interchangeably to examine plant-environment interactions, and recommend wider surveys investigating the relationship between bulk and CS $\delta^{13}C$ values across a range of plant functional types. We theorise that inter- and intra-species

variation in bulk $\delta^{13}C$ values is driven by environmental factors such as water availability and salinity. For CS values, post-photosynthetic exchange appears to be an important control on $^{13}C/^{12}C$ composition, with differences in the production of osmolytes for protection against high salinities appearing to offer an explanation for the difference in the relationship between bulk and CS $\delta^{13}C$ values across the growing seasons observed for monocots and dicots/succulents. As the production of protective compounds is a widely utilised defence against a range of environmental stresses, we recommend future studies investigate this area further, through linked analysis of the concentration and carbon isotope composition of these compounds, alongside measurement of *n*-alkane and bulk tissue $^{13}C/^{12}C$.

Despite the increased variation in biomarker carbon isotope values (relative to bulk tissue), we find that the percentage uncertainty associated with using *n*-alkane $\delta^{13}C$ values to reconstruct C_3 vs. C_4 in this temperate saltmarsh is ~11%, well below previous estimates. We therefore conclude that where appropriate, future studies seeking to use leaf wax biomarker values to reconstruct C_3 and C_4 vegetation could limit the percentage uncertainty of such biomarker-based vegetation reconstructions by using extant plants growing at a specific site or depositional environment to inform interpretation of fossil plant *n*-alkyl lipids.

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Table captions:

Table 1

Sampling of plants from Stiffkey saltmarsh across the 2011 and 2012 growing seasons

Table 2

Carbon isotope composition of *n*-alkanes and bulk tissue from plants sampled during 2011

Table 3

Carbon isotope composition of *n*-alkanes and bulk tissue from plants sampled during 2012

Table 4

n-Alkane concentrations (in μg/g of dry plant material) from plants sampled during 2011

Table 5

n-Alkane concentrations (in μg/g of dry plant material) from plants sampled during 2012

Figure captions:

Fig. 1. Stiffkey saltmarsh (inset), Norfolk, UK. Grey circles on the inset show the three sampling locations within the marsh, abbreviations: LM – low marsh; R – Ridge; UM – upper marsh.

Fig. 2. Range in carbon isotope composition among species at Stiffkey saltmarsh across the 2011 and 2012 growing seasons. Abbreviations: *AP, Atriplex portulacoides; EA, Elytrigia atherica; LV, Limonium vulgare; PA,*

Phragmites australis; SA, Spartina anglica (grey circles); SE, Salicornia europaea; SV, Suaeda vera.

Fig. 3. Seasonal trends in bulk plant leaf, *n*-C_{27}, *n*-C_{29} *n*-alkane carbon isotope ratios from all species sampled at Stiffkey saltmarsh. Sample replicates of the same species at the bulk level did not vary by more than 0.6‰, while *n*-alkane sample replicates varied by no more than 2‰. *S. europaea* and *P. australis* were not sampled in March 2012 as new growth was not observed in these species until May.

Fig. 4. Calculated seasonal variation in the apparent fractionation (*εf*) between bulk leaf and WA *n*-alkane $\delta^{13}C$ values from species sampled at Stiffkey during 2011 and 2012. Abbreviations are the same as in Fig. 2. *S. europaea* and *P. australis* were not sampled in March 2012 as new growth was not observed in these species until May.

Fig. 5. Comparison between *n*-C_{29} concentration (grey bars) and the offset between bulk and *n*-C_{29} carbon isotope values (black circles) for the 7 species sampled throughout the 2012 growing season. Abbreviations are the same as in Fig. 2.

Fig. 6. Seasonal trends in C_3 plant carbon isotope discrimination values calculated from both bulk plant leaf $\delta^{13}C$ values, and from *n*-C_{29} $\delta^{13}C$ values.

S. europaea and *P. australis* were not sampled in March 2012 as new growth was not observed in these species until May. Abbreviations are the same as in Fig. 2.

Fig. 7. Concentration of *n*-C_{27}, *n*-C_{29} and *n*-C_{31} from all C_3 species sampled at Stiffkey in 2011 and 2012. Error bars show variation between sample replicates of the same species. *S. europaea* and *P. australis* were not sampled in March 2012 as new growth was not observed in these species until May.

Fig. 8. Stacked plots showing the variability in *n*-C_{27}, *n*-C_{29} and *n*-C_{31} concentrations for the seven sampled species at Stiffkey across the 2011 and 2012 growing seasons (please see online version for a colour image). Abbreviations are the same as in Fig. 2.

Table 1
Sampling of plants from Stiffkey saltmarsh across the 2011 and 2012 growing seasons

Location	Plant Species	Plant type
LM	*Spartina anglica*	C_4 grass
LM	*Salicornia europaea*	C_3 annual succulent
R	*Elytrigia atherica*	C_3 grass
R	*Phragmites australis*	C_3 reed
R	*Suaeda vera*	C_3 evergreen succulent
R	*Atriplex portulacoides*	C_3 evergreen shrub
UM	*Limonium vulgare*	C_3 perennial herb

Table 2
Carbon isotope composition of *n*-alkanes and bulk tissue from plants sampled during 2011

Month	Location	Plant species	$\delta^{13}C$ *n*-C_{27}	AD $_{n\text{-}C27}$	$\delta^{13}C$ *n*-C_{29}	AD $_{n\text{-}C29}$	$\delta^{13}C$ *n*-C_{31}	AD $_{n\text{-}C31}$	WA*	$\delta^{13}C$ bulk	AD** $_{13C\ bulk}$	$\varepsilon f_{bulk/WA}$
June	R	*AP*	–30.5	1.1	–31.3	1.4	–32.6	1.4	–31.0	–25.7	0	–5
	R	*EA*	–36.6	0.6	–37.1	0.4	–36.2	0.6	–36.7	–28.6	0.1	–8
	UM	*LV*	–33.5	0.3	–32.7	0.3	–32.3	0.4	–32.9	–25.8	0.1	–5
	R	*PA*	–32.9	0.2	–32.9	0.4	–	–	–32.9	–25.5	0	–8
	LM	*SE*	–31.9	1.3	–31.0	2.3	–31.8	1.9	–31.8	–28.1	0	–4
	LM	*SA*	–25.1	0.3	–25.6	0.2	–24.9	0.1	–25.3	–13.9	0.2	–12
	R	*SV*	–36.7	0.2	–35.3	0.1	–35.4	–	–35.6	–29.8	0	–6
August	R	*AP*	–29.8	0.2	–31.2	0.1	–32.5	0.7	–30.5	–25.3	0	–5
	R	*EA*	–34.3	0.1	–35.2	0.1	–33.4	0.5	–34.5	–25.6	0.1	–9
	UM	*LV*	–34.3	0.2	–35	0.2	–34.6	0.3	–34.6	–26.8	0.2	–7
	R	*PA*	–33.3	0.1	–33.3	0.1	–	–	–33.2	–26.0	0	–7
	LM	*SE*	–32.7	0.3	–32.9	0.2	–33.2	–	–32.7	–25.8	0.1	–7
	LM	*SA*	–24.0	0.2	–24.7	0.1	–24.8	0.2	–24.6	–14.3	0	–10
	R	*SV*	–36.6	0.6	–35.5	0.4	–34	0.5	–35.9	–29.4	0	–7
October	R	*AP*	–30.4	0.1	–31.1	0.1	–32.2	–	–30.9	–24.9	0.1	–6
	R	*EA*	–35.2	0.3	–35.3	0.3	–34.2	0.3	–34.8	–25.9	0.2	–9
	UM	*LV*	–34.3	0.1	–34.2	0.3	–33.1	0.3	–32.6	–25.3	0.1	–8
	R	*PA*	–33.3	0.1	–33.3	0.1	–	–	–33.9	–25.8	0.1	–8
	LM	*SE*	–32.1	0.5	–32.4	1	–32.6	0.4	–32.2	–25.8	0.6	–7
	LM	*SA*	–24.3	0.1	–25.3	0.2	–26.5	1.4	–25.4	–14.2	0.4	–11
	R	*SV*	–38.2	0	–37.3	0.1	–	–	–37.6	–28.3	0.2	–9

*WA: weighted average *n* –alkane carbon isotope composition; **AD: absolute difference in carbon isotope values measured for two sample replicates of the same species

Table 3
Carbon isotope composition of *n*-alkanes and bulk tissue from plants sampled during 2012

Month	Location	Plant species	$\delta^{13}C$ *n*-C_{27}	AD $_{n-C27}$	$\delta^{13}C$ *n*-C_{29}	AD $_{n-C29}$	$\delta^{13}C$ *n*-C_{31}	AD $_{n-C31}$	WA*	$\delta^{13}C$ bulk	AD** $_{13C\,bulk}$	$\varepsilon f_{bulk/WA}$
March	R	*AP*	–31.9	0.4	–31.9	0.2	–	–	–31.9	–27.9	0.5	–4
	R	*EA*	–37.5	0.1	–36.5	0.1	–36.6	0.1	–36.6	–28.6	0	–8
	UM	*LV*	–36.2	–	–36.4	0.2	–35	0.3	–35.8	–27.1	0.5	–9
	LM	*SA*	–24.2	0.3	–25	0.4	–25	0.2	–24.7	–13.6	0	–11
	R	*SV*	–39.5	0.2	–38.5	0.3	–37.9	0.5	–38.6	–31.4	0.2	–7
May	R	*AP*	–29.9	1.2	–31.3	0.5	–31	0.8	–31.4	–27.4	0.1	–4
	R	*EA*	–38.7	0.5	–39.6	0.2	–39.3	0.2	–39	–30.7	0	–9
	UM	*LV*	–31.4	0.6	–31.1	0.7	–30.9	0.6	–31.1	–25	0.1	–6
	R	*PA*	–32.8	0.1	–34	0.2	–	–	–33.1	–26	0	–7
	LM	*SE*	–32.5	0.7	–31	0.3	–32.3	0.5	–32.2	–27.6	0	–5
	LM	*SA*	–25.9	0.2	–26.4	0.1	–	–	–26.1	–13.5	0	–13
	R	*SV*	–40.0	0.2	–37.7	0.3	–36.2	0.2	–37.9	–30.4	0.1	–8
August	R	*AP*	–31.8	0.4	–32.4	0.6	–	–	–32.0	–26	0	–5
	R	*EA*	–36.7	0.1	–37.8	0	–36.6	0.2	–37.3	–27.7	0	–10
	UM	*LV*	–32.4	0	–32.4	0.3	–32.1	0.1	–32.2	–25.2	0	–8
	R	*PA*	–32.6	0.3	–32.9	0.2	–	–	–32.6	–25.2	0	–8
	LM	*SE*	–32.8	0.2	–33.4	0.1	–33.5	0.1	–32.9	–27.5	0	–6
	LM	*SA*	–24.0	0.1	–24.7	0	–	–	–24.6	–13.0	0	–12
	R	*SV*	–37.0	0.6	–35.9	0.6	–34.4	0.6	–36.1	–29.2	0	–7
September	R	*AP*	–31.7	0.2	–32.0	0.4	–32.2	0.2	–31.8	–26.6	0.1	–5
	R	*EA*	–36.7	0.1	–37.0	0	–35.3	0	–36.2	–27.6	0	–9
	UM	*LV*	–32.8	0.4	–32.9	0.4	–32.3	0.4	–32.5	–25.5	0.1	–7
	R	*PA*	–32.6	0.3	–32.7	0.1	–	–	–32.6	–25.1	0	–8
	LM	*SE*	–32.7	0.5	–31.6	0.3	–32.1	0.5	–32.2	–26.5	0	–6

LM	*SA*	–24.5	1.2	–25.6	0.5	–25.3	0.1	–25.2	–13.4	0	–12
R	*SV*	–37.7	0.3	–36.4	0.2	–	–	–36.8	–29	0.1	–8

*WA: weighted average *n*-alkane carbon isotope composition; **AD: absolute difference in carbon isotope values measured for two sample replicates of the same species,

Table 4
***n*-Alkane concentrations (in µg/g of dry plant material) for species sampled in 2011**

Month	Location	Plant Species	C_{27}	C_{29}	C_{31}
June	R	*AP*	56	46	7
	R	*EA*	21	138	86
	UM	*LV*	676	827	538
	R	*PA*	28	43	3
	LM	*SE*	4	6	4
	LM	*SA*	34	118	31
	R	*SV*	49	93	44
August	R	*AP*	41	37	6
	R	*EA*	5	25	23
	UM	*LV*	54	190	219
	R	*PA*	26	37	1
	LM	*SE*	1	1	1
	LM	*SA*	19	109	39
	R	*SV*	66	53	22
October	R	*AP*	29	26	4
	R	*EA*	26	111	91
	UM	*LV*	78	206	137
	R	*PA*	30	40	2
	LM	*SE*	10	5	2
	LM	*SA*	20	105	30
	R	*SV*	70	54	11

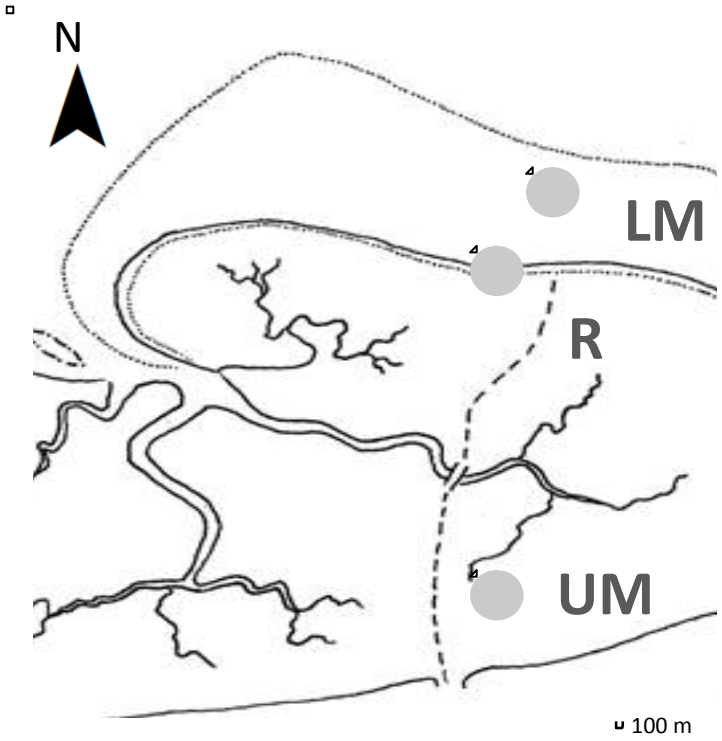
Table 5
***n*-Alkane concentrations (in µg/g of dry plant material) for species sampled in 2012**

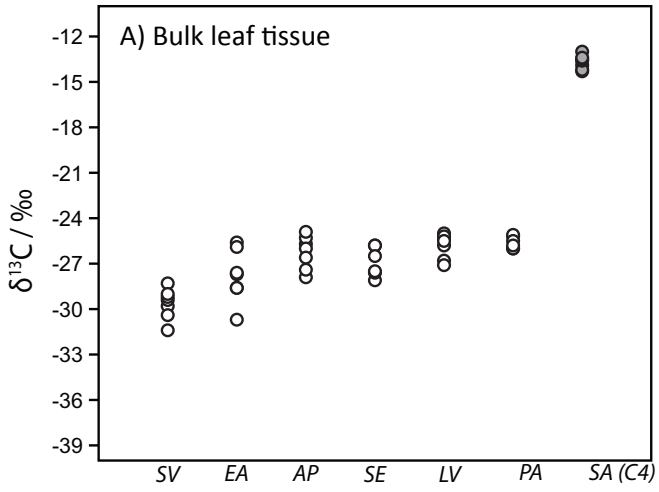
Month	Location	Plant Species	C_{27}	C_{29}	C_{31}
March	R	*AP*	127	118	28
	R	*EA*	107	599	373
	UM	*LV*	6	190	386
	LM	*SA*	20	76	24
	R	*SV*	142	201	103
May	R	*AP*	67	52	8
	R	*EA*	16	97	71
	UM	*LV*	228	391	295
	R	*PA*	6	6	0
	LM	*SE*	1	1	1
	LM	*SA*	28	49	10
	R	*SV*	43	49	31
August	R	*AP*	56	43	7
	R	*EA*	13	93	49
	UM	*LV*	134	222	222
	R	*PA*	15	17	1
	LM	*SE*	1	1	1
	LM	*SA*	5	23	5
	R	*SV*	68	62	22
September	R	*AP*	39	37	8
	R	*EA*	17	57	48
	UM	*LV*	160	311	294
	R	*PA*	24	28	2
	LM	*SE*	4	4	2
	LM	*SA*	14	71	22
	R	*SV*	66	44	11

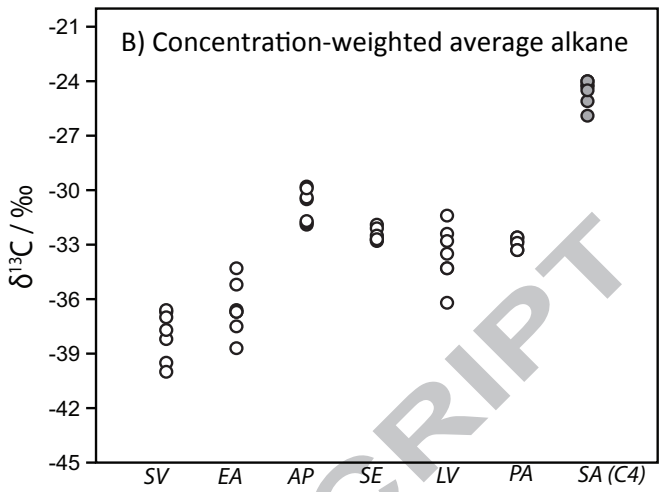
Highlights:

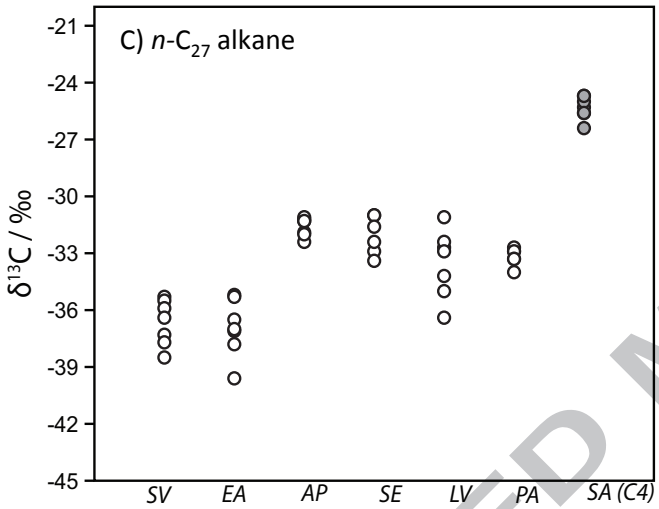
- Seasonal variation in C_3 *n*-alkane $\delta^{13}C$ (< 10‰) exceeded that in bulk tissue (< 7‰)
- Seasonal trends in bulk and *n*-alkane $\delta^{13}C$ diverge for C_3 dicots and succulents
- Apparent fractionation between plant bulk and *n*-alkane $\delta^{13}C$ reached 13‰
- Plants varied by > 2 orders of magnitude in the amount of *n*-alkanes they produced
- C_3/C_4 reconstruction in temperate salt marshes has a potential uncertainty of ~11%.

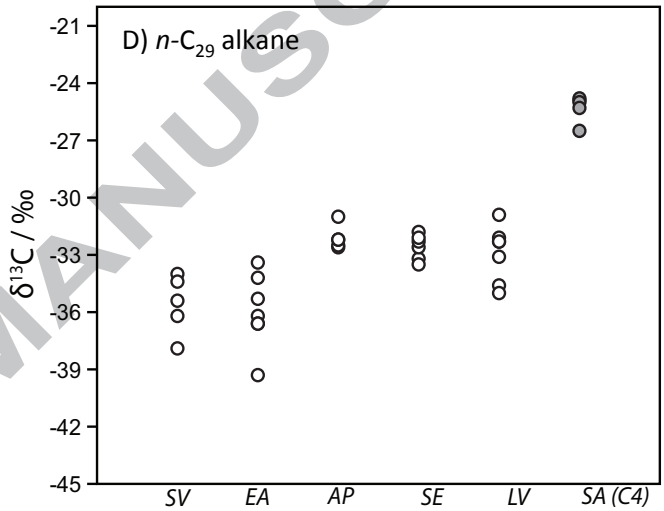

100 km

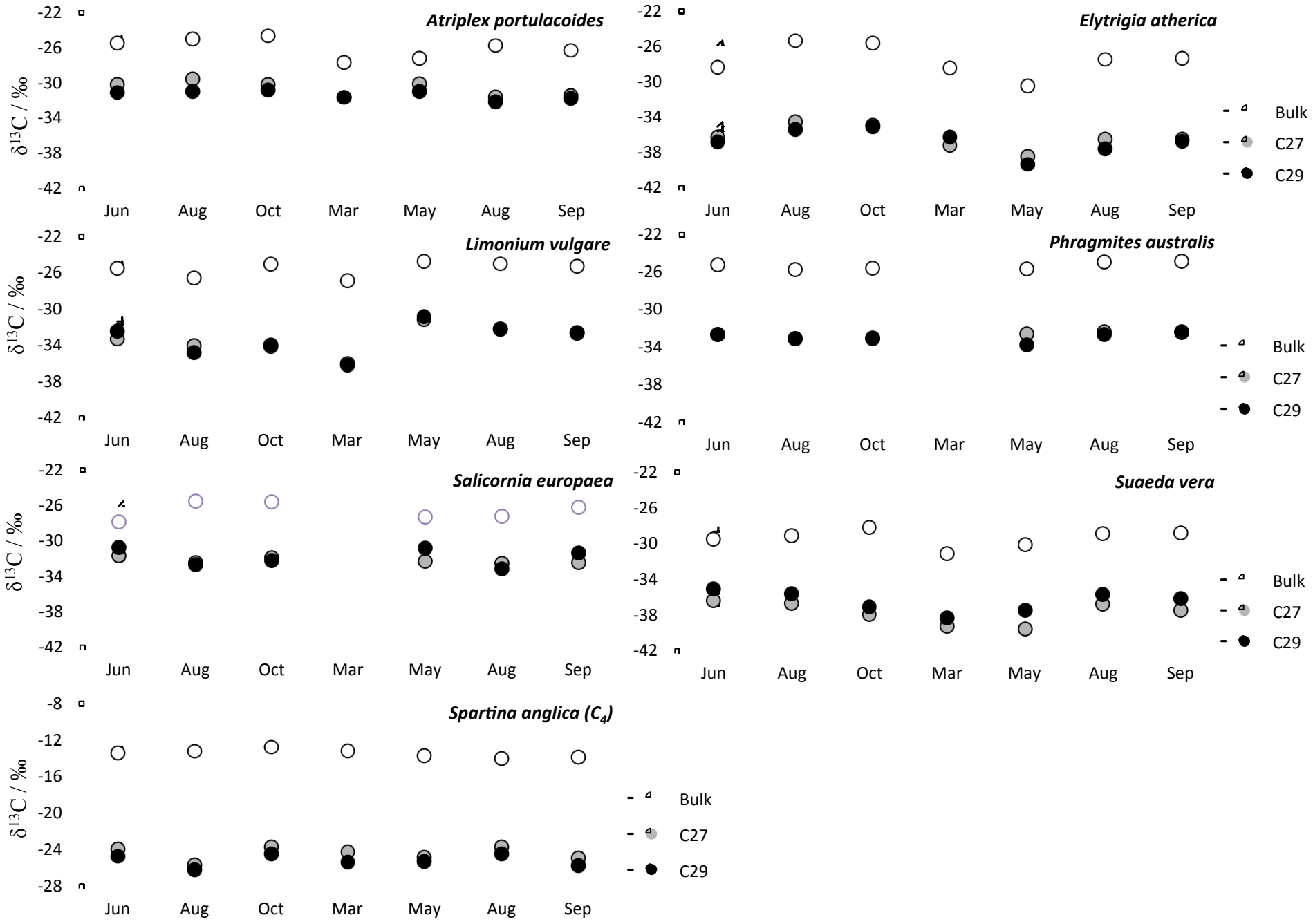

N
LM
R
UM
100 m

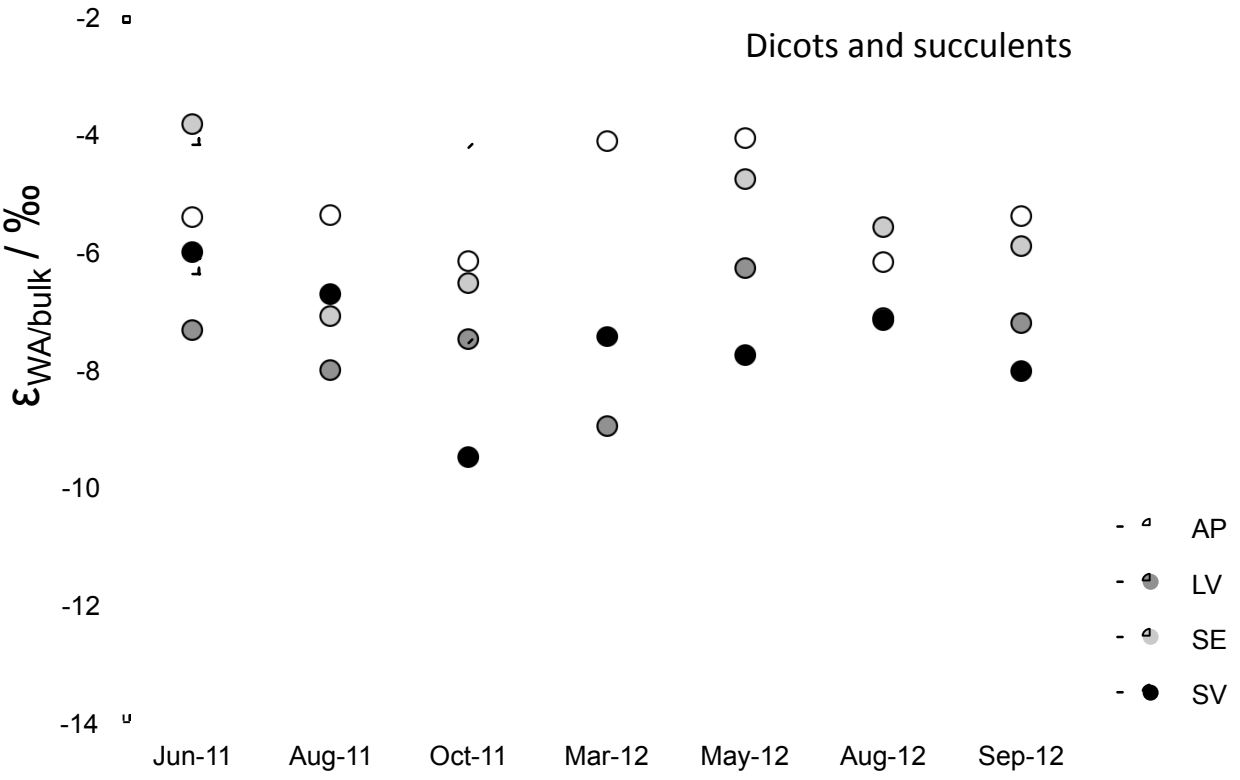


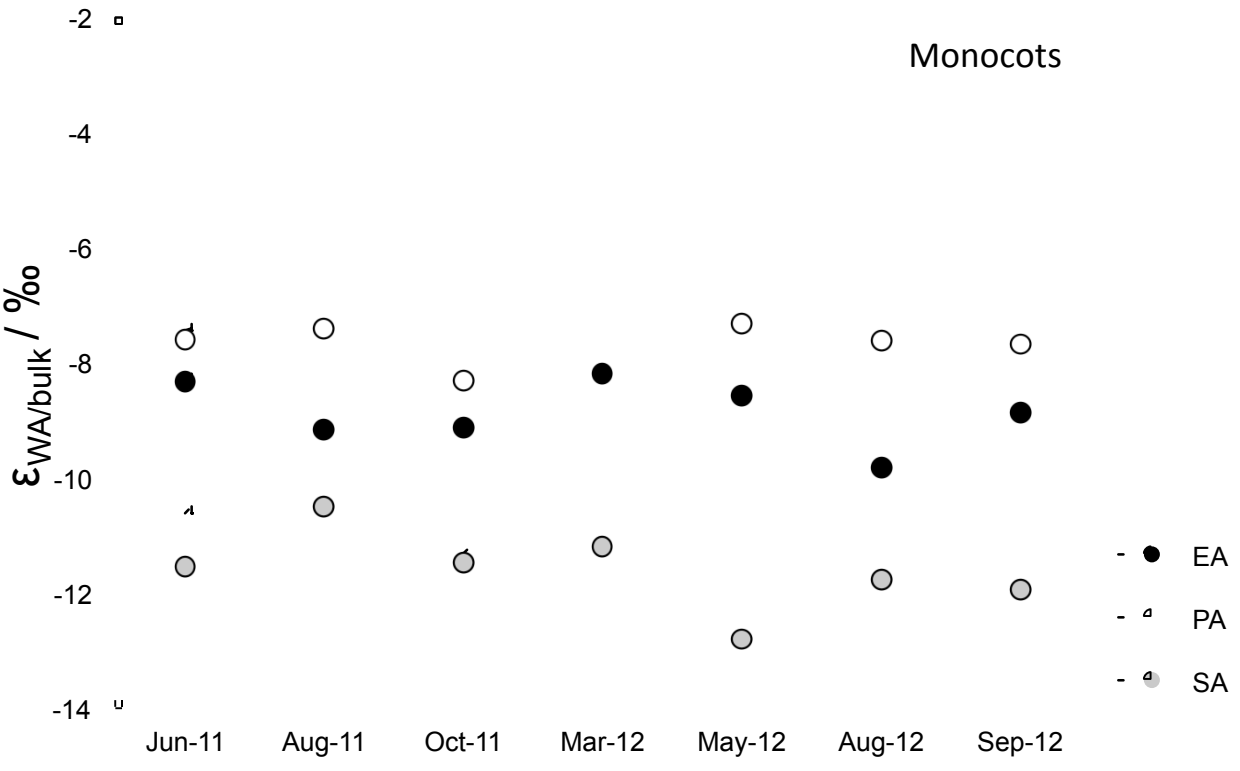







Atriplex portulacoides
Elytrigia atherica
Limonium vulgare
Phragmites australis
Salicornia europaea
Suaeda vera
Spartina anglica (C4)
δ13C / ‰
Jun
Aug
Oct
Mar
May
Aug
Sep
Bulk
C27
C29


Dicots and succulents
$\varepsilon_{WA/bulk}$ / ‰
-2
-4
-6
-8
-10
-12
-14
Jun-11
Aug-11
Oct-11
Mar-12
May-12
Aug-12
Sep-12
AP
LV
SE
SV

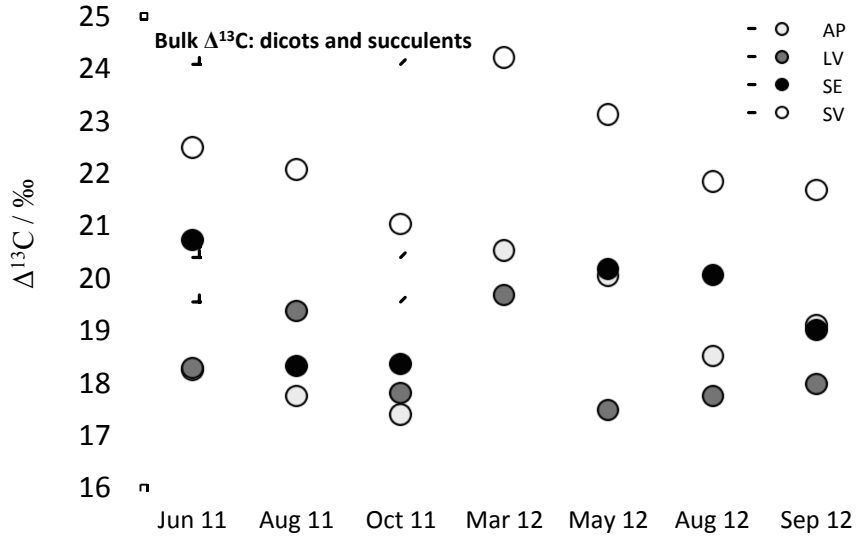

Monocots
$\varepsilon_{WA/bulk}$ / ‰
-2
-4
-6
-8
-10
-12
-14
Jun-11
Aug-11
Oct-11
Mar-12
May-12
Aug-12
Sep-12
EA
PA
SA

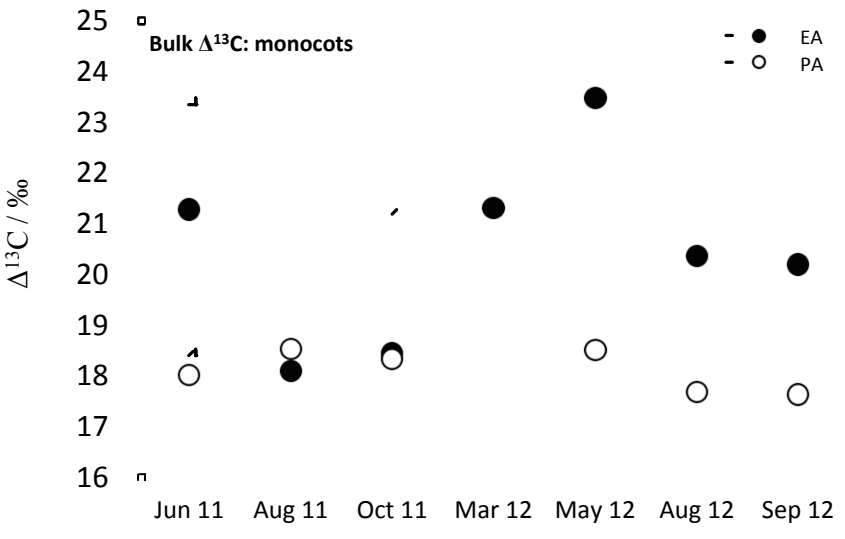
µg per g dry mass n-C29
δ13C n-C29 - δ13C bulk / ‰
0
100
200
300
400
500
600
700
0
-2
-4
-6
-8
-10
-12
AP
SV
LV
EA

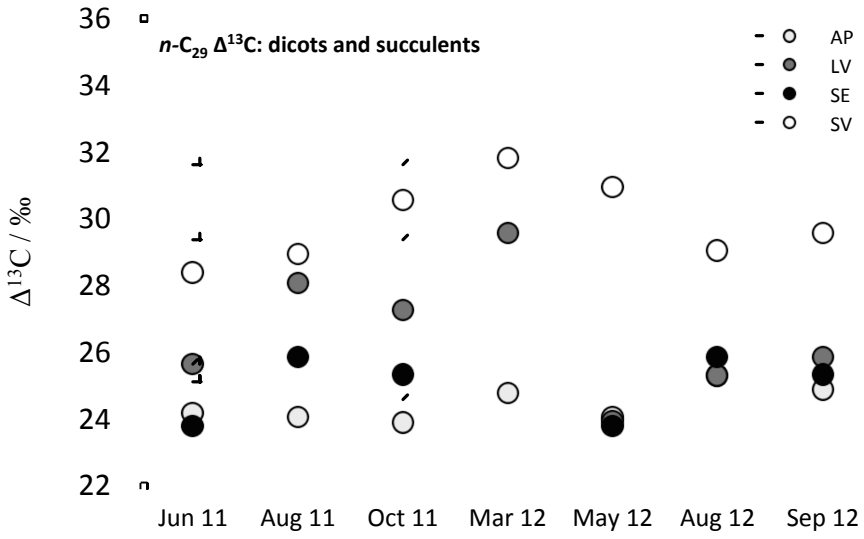
µg per g dry mass n-C29
δ13C n-C29 - δ13C bulk / ‰
0
100
200
300
400
500
600
700
0
-2
-4
-6
-8
-10
-12
SE
PA
SV
AP
EA
LV

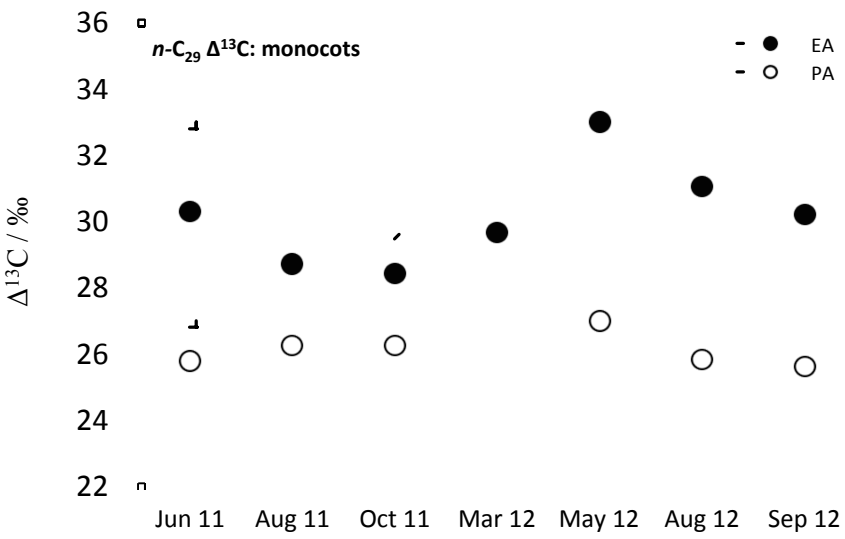
µg per g dry mass n-C29
δ13C n-C29 - δ13C bulk / ‰
0
100
200
300
400
500
600
700
0
-2
-4
-6
-8
-10
-12
SE
SV
PA
AP
EA
LV

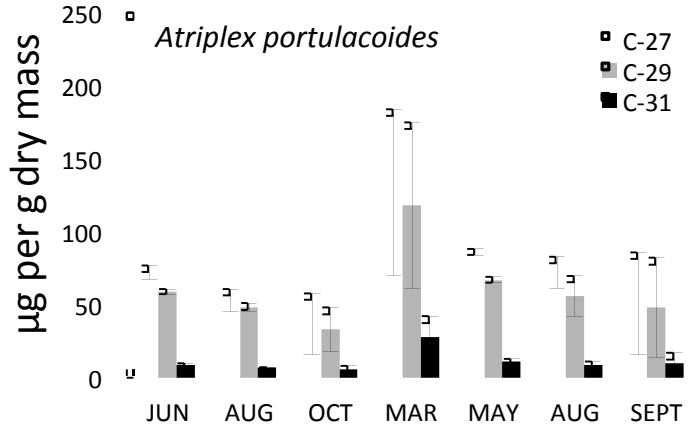
µg per g dry mass n-C29
δ13C n-C29 - δ13C bulk / ‰
0
100
200
300
400
500
600
700
0
-2
-4
-6
-8
-10
-12
SE
PA
AP
SV
EA
LV

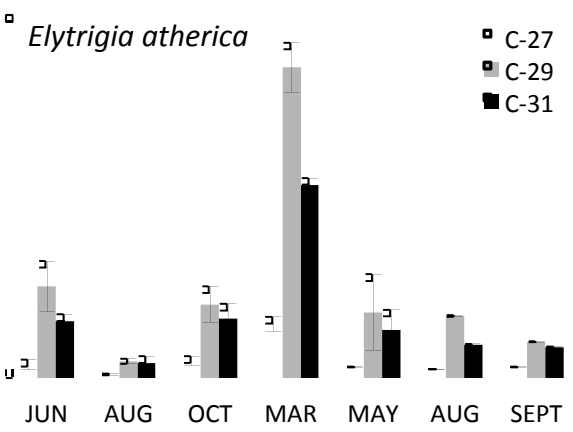

Bulk Δ13C: dicots and succulents
AP
LV
SE
SV
Δ13C / ‰
25
24
23
22
21
20
19
18
17
16
Jun 11
Aug 11
Oct 11
Mar 12
May 12
Aug 12
Sep 12

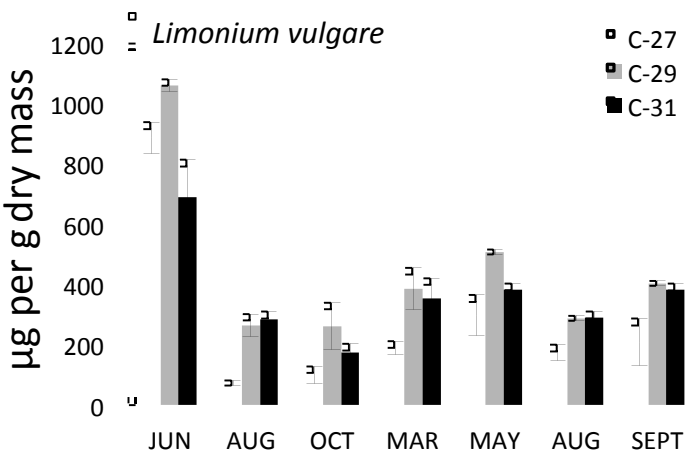

Bulk Δ13C: monocots
EA
PA
Δ13C / ‰
25
24
23
22
21
20
19
18
17
16
Jun 11
Aug 11
Oct 11
Mar 12
May 12
Aug 12
Sep 12

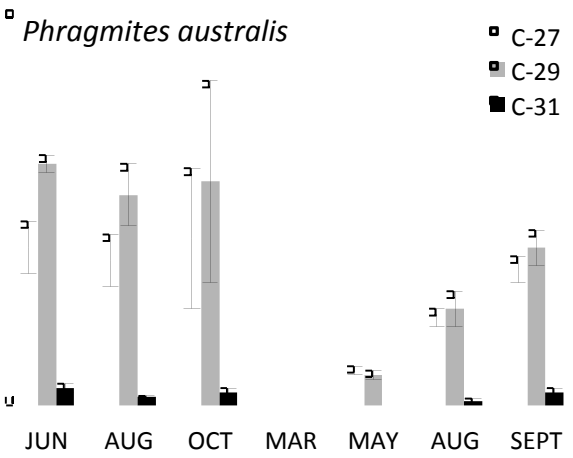

n-C29 Δ13C: dicots and succulents
AP
LV
SE
SV
Δ13C / ‰
36
34
32
30
28
26
24
22
Jun 11
Aug 11
Oct 11
Mar 12
May 12
Aug 12
Sep 12

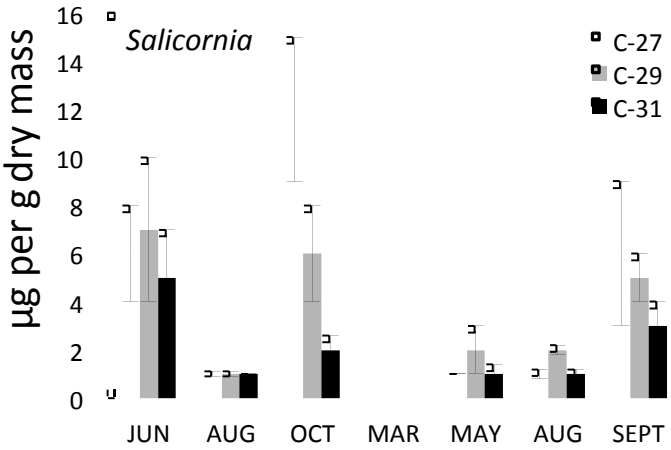

n-C29 Δ13C: monocots
EA
PA
Δ13C / ‰
36
34
32
30
28
26
24
22
Jun 11
Aug 11
Oct 11
Mar 12
May 12
Aug 12
Sep 12

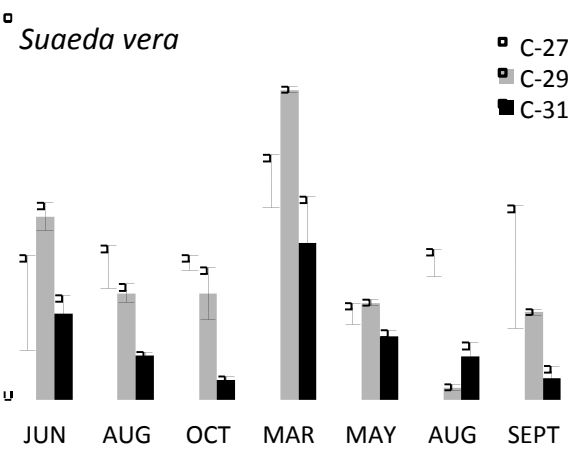

Atriplex portulacoides
µg per g dry mass
C-27
C-29
C-31
250
200
150
100
50
0
JUN
AUG
OCT
MAR
MAY
AUG
SEPT

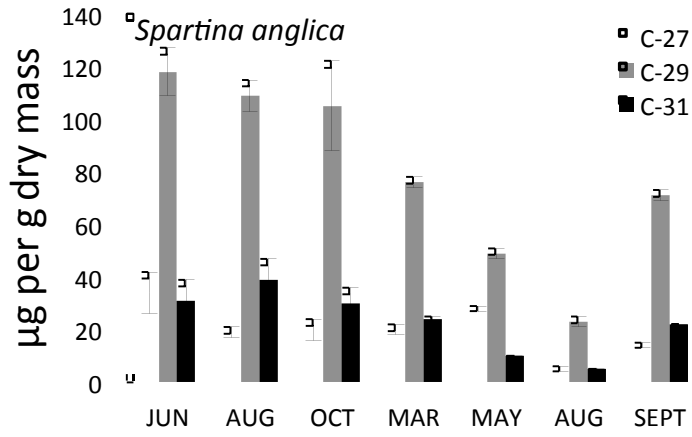

Elytrigia atherica
C-27
C-29
C-31
700
600
500
400
300
200
100
0
JUN
AUG
OCT
MAR
MAY
AUG
SEPT

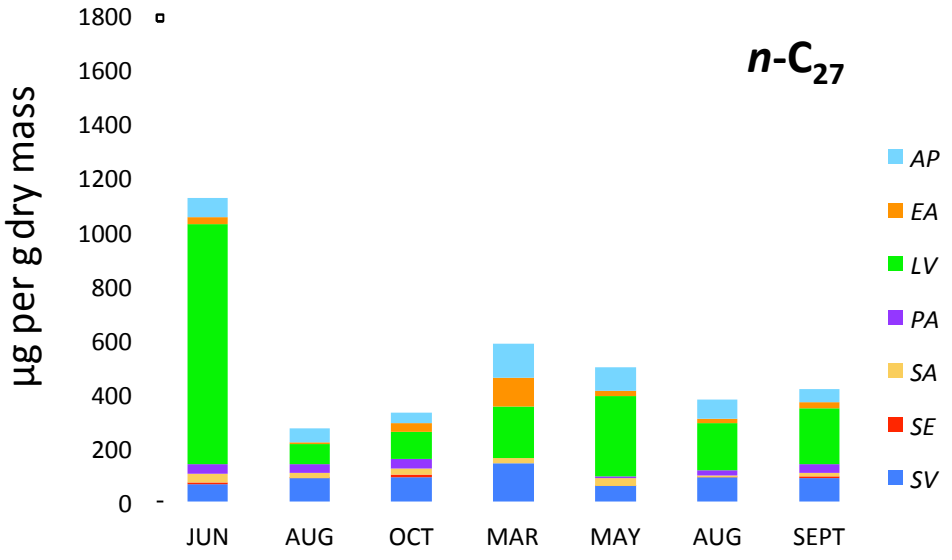

Limonium vulgare
µg per g dry mass
C-27
C-29
C-31
1200
1000
800
600
400
200
0
JUN
AUG
OCT
MAR
MAY
AUG
SEPT

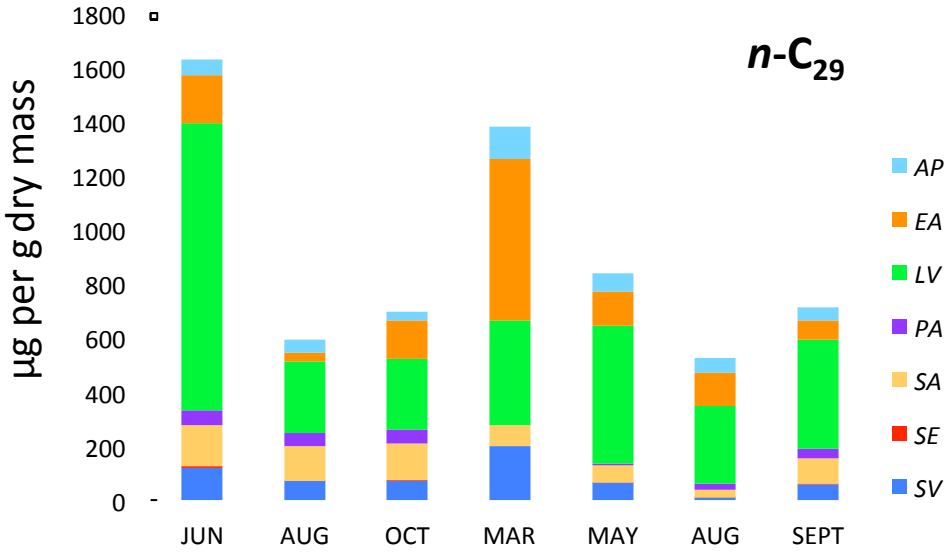

Phragmites australis
C-27
C-29
C-31
90
80
70
60
50
40
30
20
10
0
JUN
AUG
OCT
MAR
MAY
AUG
SEPT

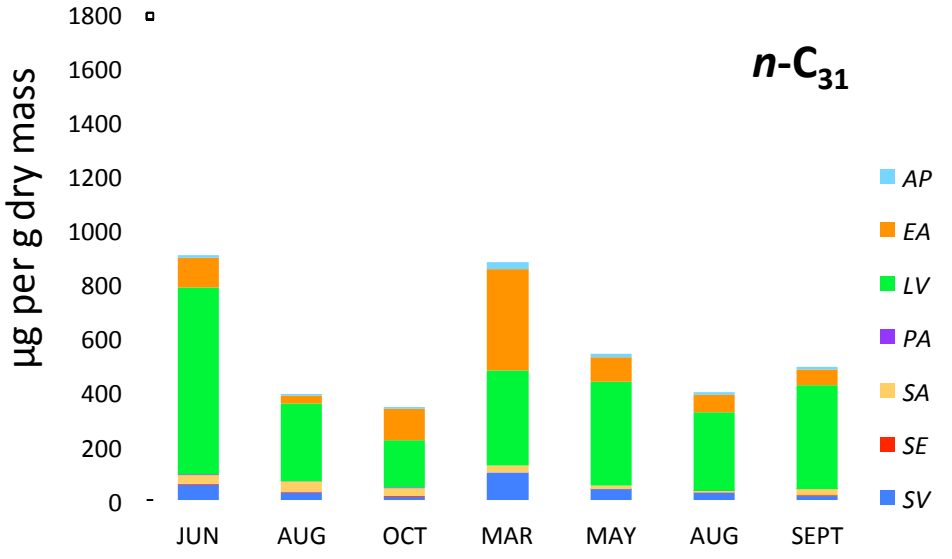

Salicornia
µg per g dry mass
C-27
C-29
C-31
16
14
12
10
8
6
4
2
0
JUN
AUG
OCT
MAR
MAY
AUG
SEPT


Suaeda vera
C-27
C-29
C-31
250
200
150
100
50
0
JUN
AUG
OCT
MAR
MAY
AUG
SEPT


Spartina anglica
µg per g dry mass
C-27
C-29
C-31
140
120
100
80
60
40
20
0
JUN
AUG
OCT
MAR
MAY
AUG
SEPT


n-C27
µg per g dry mass
1800
1600
1400
1200
1000
800
600
400
200
0
JUN
AUG
OCT
MAR
MAY
AUG
SEPT
AP
EA
LV
PA
SA
SE
SV


n-C29
µg per g dry mass
1800
1600
1400
1200
1000
800
600
400
200
0
JUN
AUG
OCT
MAR
MAY
AUG
SEPT
AP
EA
LV
PA
SA
SE
SV


n-C31
µg per g dry mass
1800
1600
1400
1200
1000
800
600
400
200
0
JUN
AUG
OCT
MAR
MAY
AUG
SEPT
AP
EA
LV
PA
SA
SE
SV