

Understanding 2H/1H systematics of leaf wax n-alkanes in coastal plants at Stiffkey saltmarsh, Norfolk, UK

Article

Accepted Version

Eley, Y., Dawson, L., Black, S. ORCID: <https://orcid.org/0000-0003-1396-4821>, Andrews, J. and Pedentchouk, N. (2014) Understanding 2H/1H systematics of leaf wax n-alkanes in coastal plants at Stiffkey saltmarsh, Norfolk, UK. *Geochimica Et Cosmochimica Acta*, 128. pp. 13-28. ISSN 0016-7037 doi: 10.1016/j.gca.2013.11.045 Available at <https://centaur.reading.ac.uk/44874/>

It is advisable to refer to the publisher's version if you intend to cite from the work. See [Guidance on citing](#).

To link to this article DOI: <http://dx.doi.org/10.1016/j.gca.2013.11.045>

Publisher: Elsevier

All outputs in CentAUR are protected by Intellectual Property Rights law, including copyright law. Copyright and IPR is retained by the creators or other copyright holders. Terms and conditions for use of this material are defined in the [End User Agreement](#).

www.reading.ac.uk/centaur

CentAUR

Central Archive at the University of Reading

Reading's research outputs online

**Understanding $^2\text{H}/^1\text{H}$ systematics of leaf wax *n*-alkanes in coastal plants at Stiffkey
saltmarsh, Norfolk, UK**

Yvette Eley^{*1}, Lorna Dawson², Stuart Black³, Julian Andrews¹, and Nikolai Pedentchouk¹

¹School of Environmental Sciences, University of East Anglia, Norwich, NR4 7TJ, UK

²The James Hutton Institute, Craigiebuckler, Aberdeen, AB15 8QH, UK

³Department of Archaeology, School of Archaeology, Geography and Environmental Science,
University of Reading, Whiteknights, Shinfield Road, Reading, Berkshire RG6 6AB, UK

* Corresponding author email address: y.eley@uea.ac.uk

Tel: +44 (0)1603 593990

Fax: +44 (0)1603 591327

ABSTRACT

Interpretation of sedimentary *n*-alkyl lipid $\delta^2\text{H}$ data is complicated by a limited understanding of factors controlling interspecies variation in biomarker $^2\text{H}/^1\text{H}$ composition. To distinguish between the effects of interrelated environmental, physical and biochemical controls on the hydrogen isotope composition of *n*-alkyl lipids, we conducted linked $\delta^2\text{H}$ analyses of soil water, xylem water, leaf water and *n*-alkanes from a range of C_3 and C_4 plants growing at a UK saltmarsh (i) across multiple sampling sites, (ii) throughout the 2012 growing season, and (iii) at different times of the day. Soil waters varied isotopically by up to 35‰ depending on marsh sub-environment, and exhibited site-specific seasonal shifts in $\delta^2\text{H}$ up to a maximum of 31‰. Maximum interspecies variation in xylem water was 38‰, while leaf waters differed seasonally by a maximum of 29‰. Leaf wax *n*-alkane $^2\text{H}/^1\text{H}$, however, consistently varied by over 100‰ throughout the 2012 growth season, resulting in an interspecies range in the $\epsilon_{\text{wax/leaf water}}$ values of -79 to -227‰. From the discrepancy in the magnitude of these isotopic differences, we conclude that mechanisms driving variation in the $^2\text{H}/^1\text{H}$ composition of leaf water, including (i) spatial changes in soil water $^2\text{H}/^1\text{H}$, (ii) temporal changes in soil water $^2\text{H}/^1\text{H}$, (iii) differences in xylem water $^2\text{H}/^1\text{H}$, and (iv) differences in leaf water evaporative ^2H -enrichment due to varied plant life forms, cannot explain the range of *n*-alkane $\delta^2\text{H}$ values we observed. Results from this study suggests that accurate reconstructions of palaeoclimate regimes from sedimentary *n*-alkane $\delta^2\text{H}$ require further research to constrain those biological mechanisms influencing species-specific differences in $^2\text{H}/^1\text{H}$ fractionation during lipid biosynthesis, in particular where plants have developed biochemical adaptations to water-stressed conditions. Understanding how these mechanisms interact with environmental conditions will be crucial to ensure accurate interpretation of hydrogen isotope signals from the geological record.

38

39

1. INTRODUCTION

40

41 The use of *n*-alkyl lipids to investigate palaeoclimatological and palaeohydrological regimes
42 has received considerable attention in the last decade as a result of initial analytical advances
43 in compound-specific stable hydrogen isotope methodology (e.g. Hilkert et al., 1999; Meier-
44 Augenstein, 1999). Of particular importance for the utility of these compounds as
45 palaeoclimate proxies is the relationship between their $^2\text{H}/^1\text{H}$ composition and that of
46 environmental water. Previous studies have demonstrated a link between the $\delta^2\text{H}$ values of *n*-
47 alkyl lipids from modern plants and source water across geographically and climatically
48 diverse transects (Huang et al., 2002; Sachse et al., 2004, 2006; Garcin et al., 2012; Tipple
49 and Pagani, 2013; Kahmen et al., 2013b). However, when leaf wax biomarkers from a range
50 of plant species from the same biosynthetic group at individual locations are considered,
51 significant variation in the $\delta^2\text{H}$ values of *n*-alkyl lipids – of up to 80‰ – have been observed
52 (Sachse et al., 2006; Hou et al., 2007; Pedentchouk et al., 2008; Feakins and Sessions, 2010).

53

54 Palaeoclimatic reconstructions of source water isotopic composition (Pagani et al., 2006;
55 Tierney et al., 2008) and moisture availability and aridity (Scheffuß et al., 2005; Leider et al.,
56 2013) have often implicitly and/or explicitly relied on the assumption that the biosynthetic
57 $^2\text{H}/^1\text{H}$ fractionation that takes place between the intracellular water and lipids within the plant
58 is relatively invariant within C_3 and C_4 plant groups. The magnitude of variability in the $\delta^2\text{H}$
59 values of *n*-alkyl lipids among plant species growing at the same geographical location
60 suggests, however, that this assumption may not necessarily be valid. Interpretation of
61 sedimentary *n*-alkyl $\delta^2\text{H}$ data is further complicated by limited understanding of the reasons
62 for this large interspecies variability. Sachse et al. (2012) provided a comprehensive review

of the current state of knowledge regarding the factors that control hydrogen isotope composition of lipid biomarkers in photosynthetic organisms. This review highlighted the importance of both physical (mainly through influencing intracellular water $^2\text{H}/^1\text{H}$) and biochemical mechanisms in controlling $^2\text{H}/^1\text{H}$ composition of photosynthates. However, the relative importance of these separate but interrelated controls remains largely unexplored, particularly when morphologically and biochemically distinct plant species growing in a natural environment are considered.

Previous research has mainly focused on using empirical and modelling studies to investigate various physical processes that control source and intracellular water. First, there were studies (e.g. Hou et al, 2007; Pedentchouk et al, 2008) in which a range of plants were considered, but coupled leaf water and *n*-alkane $^2\text{H}/^1\text{H}$ measurements were not conducted. Instead, these studies relied on isotopic measurements of environmental water and leaf wax *n*-alkyl compounds, and any differences in $^2\text{H}/^1\text{H}$ fractionation were explained by reference to the physical processes that controlled the movement of water molecules inside, outside and within the leaf according to leaf-water models (Farquhar and Lloyd, 1993; Barbour et al., 2000; Barbour et al., 2004). The implicit assumption of these models (initially developed for understanding oxygen isotope systematics of plant water) is that they can fully describe hydrogen isotope systematics of leaf water, and thus also account for the differences in the $\delta^2\text{H}$ values of leaf wax lipids among different species. The lack of actual measurements of leaf water isotopic composition, however, prevents such studies from evaluating the relative importance of physical and biochemical factors that control leaf water and biosynthate $^2\text{H}/^1\text{H}$ signatures.

Other studies have focused on the analysis of modelled and/or empirical leaf water and *n*-alkyl lipid $^2\text{H}/^1\text{H}$ compositions to avoid the limitations inherent in the above approach. McInerney et al. (2011) examined the impact of relative humidity on leaf wax $\delta^2\text{H}$ by analysing *n*-alkanes from grasses grown both in controlled environmental chambers and across a range of climatically different field sites. Modelled leaf water $\delta^2\text{H}$ values, however, were more positive than would have been expected from empirical *n*-alkane $\delta^2\text{H}$ data. McInerney et al. (2011) suggested that ^2H -enriched leaf waters were not the biosynthetic precursor for leaf wax synthesis, as the best correlation between source water and lipid $\delta^2\text{H}$ values was obtained though using 100% xylem water. The potential for biochemical mechanisms to explain differences in fractionation between C_3 and C_4 plants was mentioned, but the design of the study did not allow for assessment of its relative importance. Sachse et al. (2010) also focused on monocot species, analysing field-grown barley (*Hordeum vulgare*) across one growing season. This study found a correlation between midday leaf water and *n*- C_{31} alkane $\delta^2\text{H}$ values. However, their model, which assumed a 1:1 relationship between leaf water (source) and leaf wax (product), overestimated ^2H -enrichment of the *n*- C_{31} alkane. The authors proposed that this discrepancy could be due to a ^2H -depleted pool of water used during biosynthesis, which may have originated from spatial inhomogeneity in ^2H -enrichment along the length of a leaf. This study did not address the question of whether biochemical mechanisms might explain the lack of a 1:1 relationship between source water and *n*-alkane $^2\text{H}/^1\text{H}$.

The potential for biochemical processes to influence leaf wax $^2\text{H}/^1\text{H}$ has been considered previously in limited circumstances. Kahmen et al. (2013) investigated whether evaporative ^2H -enrichment in leaf water was recorded in the leaf waxes of five angiosperm species grown under controlled growth chamber conditions. The results of this study suggested that the

influence of evaporative ^2H -enrichment was species-specific; with 18 to 68% of the leaf water ^2H -enrichment reflected in *n*-alkanes. However, interspecies variation of up to 65‰ was observed in $^2\text{H}/^1\text{H}$ fractionation between xylem water and *n*-alkanes. This range in fractionation could not be attributed to differences in measured leaf water evaporative ^2H -enrichment among the studied species. The authors, therefore, theorised that species-specific variation in NADPH sources used for lipid biosynthesis could have been the reason for this variation. Sessions (2006) studied seasonal shifts in the C_4 saltmarsh grass *Spartina alterniflora*, growing in seawater, which were assumed to have the same isotopic composition throughout the sampling period. The relative ^2H -depletion in lipid $^2\text{H}/^1\text{H}$ observed during the summer months – contrary to the anticipated ^2H -enrichment in summer – was interpreted as a change in the organic substrate used for lipid biosynthesis, i.e. current photosynthate in summer, versus stored carbohydrates during the winter. Feakins and Sessions (2010) considered whether changes in the source of biosynthates influenced species-specific variation in $^2\text{H}/^1\text{H}$ among CAM plants. Hydrogen isotope fractionation between source water and *n*-alkanes differed by 92‰ among species. However, the authors had not measured xylem or leaf water $\delta^2\text{H}$ as part of this study, but theorised that these differences may have arisen from metabolic moderation of fractionation between leaf water and leaf wax by using a percentage of NADPH generated from heterotrophic pathways for lipid biosynthesis.

Remarkably, the inadequacy of relying solely on physical mechanisms to explain leaf water empirical $\delta^2\text{H}$ data was shown by Shu et al. (2008), who modelled leaf water oxygen and hydrogen isotope compositions along the length of a pine needle. Even though their model could describe along-leaf variation in empirical $\delta^{18}\text{O}$ data, it could not do it for $\delta^2\text{H}$ data. The authors proposed that this discrepancy was due to the fact that “certain unknown biological processes may not have been incorporated into our 2D model ... it calls for a re-evaluation of

all the other models for hydrogen isotopic simulations of leaf water since they too lack these processes". The results of this study implied that interpretation of both leaf water and *n*-alkyl lipid $\delta^2\text{H}$ values required a new approach that integrated $^2\text{H}/^1\text{H}$ fractionation during physical processes that control water movement in, out and within the leaf with that which takes place at various stages of photosynthesis.

As a result of all the previous research we can therefore hypothesize that if interspecies differences in the $^2\text{H}/^1\text{H}$ composition of leaf wax lipids are driven primarily by differences in the isotopic composition of leaf water, there are several theoretical scenarios that may account for the observed variability among plant species growing at the same site. These include: (i) differences in the isotopic composition of soil water among site sub-environments; (ii) differences in the isotopic composition of soil water throughout the growth season; (iii) interspecies differences in xylem water reflecting root uptake of soil water and transport to the site of evaporation in the leaf, and (iv) interspecies differences in the isotopic composition of leaf water among plant life forms due to differences in leaf structure. The focus of this paper is to test all of these scenarios and to evaluate whether they provide a comprehensive explanation for differences in the $^2\text{H}/^1\text{H}$ composition of lipids from a range of C_3 and C_4 plant species (grasses, succulents, evergreens and perennial herbs) sampled at Stiffkey salt marsh, Norfolk, UK across the entire growing season from March to September in 2012. The broad range of plant life forms was specifically chosen due to (a) their gross variation in leaf morphology, and (b) their well-studied differences in biochemical adaptations to their environment, which provided an ideal platform to test the relative importance of physical and biochemical mechanisms in explaining interspecies variation in the $\delta^2\text{H}$ values of leaf wax *n*-alkanes in terrestrial plant species growing in a geographically restricted natural environment.

In this study, we focus on a saltmarsh environment at the land/sea divide. These ecosystems contribute significant amounts of organic material to the marine environment (Mitsch and Gosselink, 2000). Indeed, globally saltmarshes are known to have higher levels of primary production than other coastal biomes such as mangroves, and greatly exceed the productivity of grasslands, cultivated plant communities and forest ecosystems (Mitsch and Gosselink, 2000; Richardson, 2000), with ~50% of organic carbon in ocean sediments being derived from vegetated sedimentary environments (Duarte et al., 2005). Findings from this study will therefore have important implications for palaeoclimate reconstructions based on the $\delta^2\text{H}$ profiles of leaf wax lipids from coastal and marine sediments. In addition, biochemical adaptations employed by the selected species at Stiffkey to ameliorate water stress are not unique to saltmarsh settings - other xeromorphic plant species growing in a variety of other water stressed habitats such as arid regions, are also known to make use of similar biochemical responses to maintain their osmotic potential (Bohnert and Jensen, 1996), and thus the conclusions can be translated to such other environments. Understanding the relative importance of biochemistry in controlling the hydrogen isotope composition of leaf wax biomarkers in plant biochemical mechanisms is therefore important for helping in the reconstruction of past climates across a range of different biomes. The data presented here thus allows us to make far-reaching inferences regarding the interaction between physical and biochemical mechanisms across a wide variety of plant life forms.

2. STUDY LOCATIONS AND SAMPLING METHODS

2.1 Study location

Stiffkey marsh is typical of an open coast back-barrier saltmarsh (Moeller et al., 1996; Allen, 2000) (Fig. 1). The site can be divided into ecologically distinct zones. The low marsh (LM) and upper marsh (UM), defined by Jeffries (1977), are separated by a well-drained gravel and sand ridge (R, Fig. 1) formed by onshore emplacement of offshore barrier sediments (Boomer and Woodcock 1999). Seawater inundation onto the upper marsh 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). There are no rivers or streams draining onto the marsh, therefore rainwater accounts for all near-surface fresh water inputs to the site.

2.2 Surface vegetation

Stiffkey vegetation cover can be zoned according to topography and degree of tidal inundation (Jeffries, 1977; Jeffries and Perkins, 1977; Davy et al., 2011). Plant types include grasses (*Spartina anglica*, *Elytrigia atherica*, *Phragmites australis*, *Puccinella maritima*), succulents (*Suaeda vera*, *Salicornia europaea*) and dicots (*Limonium vulgare*, *Atriplex portulacoides*). The low marsh at Stiffkey, which receives regular tidal inundation, is colonised by C₄ grass *Spartina anglica*, C₃ annuals *Salicornia europaea* and *Limonium vulgare*, and occasionally the C₃ shrub *Atriplex portulacoides*. The gravel ridge supports a range of C₃ grasses such as *Elytrigia atherica*, with stands of the reed *Phragmites australis* found on the seaward side. *Suaeda vera* and *Atriplex portulacoides* also grow in ≤1 m high bushes on the ridge. *Limonium vulgare*, *Atriplex portulacoides* and *Suaeda vera* are particularly abundant in the upper marsh, however, *Spartina anglica* and *Salicornia europaea*

proliferate around lower-lying brackish pools and water-logged ground surrounding old drainage channels.

The distribution of coastal plants at Stiffkey can be explained by considering ‘Ellenberg’ values for salinity tolerance produced as part of the 1999 ‘Ecofact’ project (Hill et al., 1999). An Ellenberg rating of 0 indicates a species with no salt tolerance, whilst 9 is applied to species known to favour extremely saline conditions in which hypersalinity and salt precipitation are common. Under this classification scheme, *Spartina anglica* (7) is identified as a species of the lower salt marsh; *Salicornia europaea* (9) is a species found in extremely saline and hypersaline conditions; *Atriplex portulacoides* and *Limonium vulgare* (6) are most common in mid-level salt marshes; *Suaeda vera* (5) is found typically on the upper edges of marshes where tidal inundation does not often reach; *Elytrigia atherica* (4) is most suited to salt meadows and upper marsh environments; and *Phragmites australis* (2) is a species that can live in both saline and non-saline habitats but is more predominant in non-saline environments. Species present at the site are adapted for survival in continually damp/wet soils, with the exception of *Elytrigia atherica*, which can tolerate only moderately damp conditions (Hill et al., 1999). The selected species at Stiffkey vary in terms of the compatible solutes they use for osmoregulation and amelioration of the harsh saltmarsh conditions. The main compounds synthesised for these purposes include proteins, amino-acids and sugars/carbohydrates (Bohnert and Jensen, 1996). These biological mechanisms are important since their existence is not limited to saltmarsh plants; indeed they are also widely found in other drought tolerant species (Bohnert and Jensen, 1996).

2.3 Sampling strategy

Plant samples were collected for a pilot study in June 2011, and then also in March, May August and September during the 2012 growth season. Sampling of all species collected on each occasion took place between 12:00 and 14:00 from three sites at Stiffkey (Fig. 1). This two-hour sampling window was unavoidable as a result of high tides. In June 2011, plant species were sampled (i) by plant type (grass, succulent, perennial etc) and (ii) where possible from multiple locations within the marsh (LM, R, UM), to evaluate the relative importance of marsh sub-environment on leaf lipid $^2\text{H}/^1\text{H}$ (Supplementary Information Table 1). Our sampling strategy for the period from March to September 2012 was based upon the key findings from the initial results obtained in June 2011. The 2012 sampling focused on gross interspecies differences in hydrogen isotope fractionation between leaf wax, leaf water and environmental water across the growing season. Seven species were selected for study during the 2012 period across the three sampling sites (Supplementary Information Table 2).

In June 2011 samples were collected for paired leaf wax and leaf water analysis. During 2012, however, sampling also included soil water samples for the entire growing season. In September 2012, we sampled xylem water as well as leaves from all species between 12:00 and 14:00. In addition we collected soil, leaf and xylem water from *Elytrigia atherica*, *Suaeda vera*, and *Atriplex portulacoides* at the ridge between 7:30 and 8:00 to allow for investigation of the potential influence of diurnal shifts in xylem and leaf water on *n*-alkane $^2\text{H}/^1\text{H}$ compositions. These three species were chosen because of their close proximity to each other and because they showed the maximum range in *n*-alkane $\delta^2\text{H}$ values among species at one sampling site.

In order to ensure that samples collected were statistically representative of each species at a given location, samples for *n*-alkane or leaf water extraction were collected in triplicate.

Further, each individual analysed sample represents a composite of at least five leaves (dependant on plant leaf morphology) taken from at least three different plants at a particular sampling site. The exception to this was the succulent *Salicornia europaea*: this species has no distinct leaves but instead has green photosynthetically active jointed stems (Ellison, 1987). Samples comprising at least five green stems were collected during 2012 for both *n*-alkane and leaf water analysis from this succulent species. Samples for soil water extraction were collected in triplicate in March, May and September 2012 from the top ~10 cm of soil in each location. Stem samples were collected in triplicate for each species in September 2012; each sample represents a minimum of three stem samples of greater than 5 cm in length. Leaf, stem and soil water samples were placed directly into exetainers, capped, taped with PTFE tape in the field, and then frozen in the laboratory until water extraction. Samples for *n*-alkane analysis were dried at 40 °C for 72 hr, and then stored at room temperature in the dark prior to lipid extraction.

3. ANALYTICAL METHODS

3.1 Leaf, xylem and soil water extraction

Leaf, xylem and soil water extractions were carried out using cryogenic vacuum distillation based upon the design and operating procedure presented by West et al. (2006). Duplicates of each sample were extracted to enable consideration of a) reproducibility of the extraction method, and b) inherent intraspecies variability in leaf/xylem/soil water isotopic composition. Samples were heated to 80 °C within an evacuated glass line and water was distilled and trapped in an adjacent collection vial submerged in liquid nitrogen. Each station on the extraction line was coupled to a pressure gauge, allowing for accurate determination of

completion and monitoring of line stability during sample collection. At the commencement of each series of extractions, line vacuum pressures at all stations were consistently ≤ 5 mTorr, which exceeds the 60 mTorr recommended by West et al. (2006). All leaf, xylem and soil samples were extracted for at least 2 hr to avoid $^2\text{H}/^1\text{H}$ fractionation during distillation.

3.2 Water isotopic analysis

Hydrogen isotope signatures of extracted waters were measured using a Delta XP ThermoFisher isotope-ratio mass spectrometer interfaced with a pyrolysis TC/EA equipped with a liquid autosampler. The $\delta^2\text{H}$ values reported here are based on ten analytical replicates of each sample. The first five replicates of each sample were discarded to prevent distortion by memory effects associated with the use of liquid autosampler. The $\delta^2\text{H}$ values are expressed relative to the VSMOW scale based upon analysis of a suite of international and in-house standards analyzed in the same sequence with the water samples. Additional standards (GISP, in-house tap water) were treated as unknowns to evaluate instrument accuracy. Root mean square (RMS) errors for $^2\text{H}/^1\text{H}$ measurements of international and in-house standards were 1.0‰ ($n = 108$). During all sample and standard measurements, three reference gas pulses were passed through the mass spectrometer. Reproducibility of H_2 reference gas $\delta^2\text{H}$ values after H_3^+ correction was typically ± 0.5 ‰. Typical standard error among analytical replicates of the same sample was 4‰, while comparison of mean values for leaf and xylem sample duplicates showed that the absolute difference between them was in all cases also less than 4‰. Soil sample duplicates could not be successfully processed for all sampling intervals due to difficulties in extracting sufficient amounts of water from them for reliable stable isotope measurements (Supplementary Information Table 4). However when they were possible, mean values did not vary by more than 4‰ among sample

311 replicates. We adopted a conservative approach and assumed that level of variability for all
312 singular soil water samples presented here.

314 **3.3 *n*-Alkane extraction and identification**

315
316 Leaf wax lipids were extracted from whole leaves by sonication with HPLC grade hexane to
317 obtain the total lipid fraction. The number of leaves used varied among species, from ~3 for
318 *Phragmites australis* to > 50 for *Suaeda vera*. Samples were extracted by sonication and the
319 extract was concentrated to 1 mL under nitrogen gas using a turbovap prior to
320 chromatographic separation. Duplicates of each sample were extracted, to ensure
321 reproducibility of the extraction process, and to evaluate intraspecies variability in the leaf
322 wax signal. The hydrocarbon fraction was eluted with HPLC grade hexane during column
323 chromatography, using activated silica gel (70-230 mesh, Merck KGaA). Analysis of the
324 molecular distribution of *n*-alkanes for each species was carried out by injection into an
325 Agilent 7820A gas chromatograph equipped with a flame ionisation detector and an Agilent
326 DB-5 capillary column (30 m × 0.32 mm × 0.25 µm) (Agilent Technologies Inc., Santa Clara,
327 USA). The oven temperature was raised from 50 °C to 150 °C at 20 °C min⁻¹, and then at 8 °C
328 min⁻¹ to 320 °C (10 min). *n*-Alkanes were identified by comparison of their elution times with
329 *n*-C₁₆ to *n*-C₃₀ alkane standard (A. Schimmelmann, Indiana University). Average chain length
330 (ACL) and carbon preference index (CPI; Supplementary Information Tables 1 and 2) values
331 were calculated following the approach of Zhang et al., (2006).

333 **3.4 *n*-Alkane hydrogen isotope analysis**

The $^2\text{H}/^1\text{H}$ 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 (HTC) system operating at 1420 °C. The initial GC oven temperature was set at 50 °C, which was then raised at a rate of 30 °C min⁻¹ to 220 °C. A second temperature ramp to a final temperature of 320 °C at a rate of 6 °C min⁻¹ followed. The final temperature was held for 5 min. The $\delta^2\text{H}$ values are based on duplicate analyses of well-resolved peaks and reported on the VSMOW scale, based on in-house reference gases (H_2 , >99.995% purity, BOC) adjusted at the beginning and at the end of each sequence using a standard mixture of the *n*-C₁₆ to *n*-C₃₀ alkane standard. Root mean square (RMS) errors for $^2\text{H}/^1\text{H}$ measurements of this standard were 4.0‰ (*n* = 780). During all sample and standard measurements, six reference gas pulses were passed through the mass spectrometer. Reproducibility of H_2 reference gas $\delta^2\text{H}$ values after H_3^+ correction was $\pm 6\text{‰}$. Typical absolute differences in *n*-C₂₉ measurements between analytical replicates of the same sample did not exceed 6‰, while absolute differences in mean values among sample replicates of the same species (an indicator of intraspecies variability) was on average 4‰, with a maximum of 10-14‰ for *Atriplex portulacoides* (August 2012), *Phragmites australis* (September 2012) and *Suaeda vera* (September 2012) (Supplementary Information Table 4).

4. RESULTS

4.1 Soil water $^2\text{H}/^1\text{H}$ composition

Soil water from the sandflat was most ^2H -depleted in March (-27‰) and most ^2H -enriched in May (+2‰) (SI Table 4). Between May and September 2012, soil water from the sandflat remained constant within analytical error, varying by only 3‰. Upper marsh soil water

samples were not successfully stored for March, however, similar seasonal consistency to that observed in the sandflat was revealed when comparing the May (+2‰) and September (-2‰) soil water samples taken from this location. The greatest seasonal shift in soil water at the site was found at the ridge, where values ranged from -36‰ in March to -5‰ in September (Supplementary Information Table 4). Soil waters collected before 8:00 in September 2012 had a mean value of -21‰, indicating they were 16‰ ²H-depleted compared with samples collected between 12:00-14:00 (Supplementary Information Table 5).

4.2 Xylem water ²H/¹H composition

Xylem waters from the September 2012 sampling interval showed that stem waters were more negative than the soil waters across all sampling sites (Fig. 2). *Elytrigia atherica* had the most negative xylem water of all species sampled (-43‰), while *Limonium vulgare* had the most positive (-4‰). Total interspecies variation in xylem water δ²H was 39‰ (Supplementary Information Table 4). Xylem samples collected from *Elytrigia atherica*, *Atriplex portulacoides* and *Suaeda vera* at the ridge site in September 2012 (a) between 7:30 and 8:00, and (b) between 12:00 and 14:00, varied by no more than 2-3‰. This was lower than both analytical reproducibility (4‰) and intraspecies variability in ²H/¹H isotopic composition (4‰). The range of xylem water values among the species sampled in the early morning was 20‰, which was slightly higher than that observed among xylem water samples collected between 12:00 and 14:00 (13‰) (Supplementary Information Table 5).

4.3 Leaf water ²H/¹H composition

Leaf waters extracted from all species collected at Stiffkey in June 2011 varied by no more than 29‰. For those species sampled from multiple locations, upper marsh leaf water samples were generally more ^2H -enriched than those sampled from other locations, but the range of variation was low compared to gross interspecies differences: $\delta^2\text{H}_{\text{LW}}$ from *Atriplex* *Portulacoides* varied by 13‰ across the marsh, with the most ^2H -depleted leaf water found at the ridge site and the most ^2H -enriched in the upper marsh; $\delta^2\text{H}_{\text{LW}}$ from *Triglochin maritima* varied by 10‰ between the lower and upper marsh. Small shifts of 6‰ were observed in the evergreen succulent *Suaeda vera*, and the perennial herb *Limonium vulgare*, with the most ^2H -enriched value occurring in the upper marsh for *Suaeda* and the lower marsh for *Limonium* (Fig. 4; Supplementary Information Table 3).

Leaf water samples collected during 2012 showed a total range among all species sampled of 46‰ between the most ^2H -depleted values (-26‰, *Limonium vulgare*, March) and the most ^2H -enriched (+20‰, *Salicornia europaea*, September). Species-specific variation in leaf water $\delta^2\text{H}$ was most limited in March (6‰) and greatest in August (29‰). Leaf waters from all species were generally most ^2H -depleted in March, and ^2H -enriched in September. *Elytrigia atherica* and *Phragmites australis* were generally the most ^2H -depleted in terms of leaf water $\delta^2\text{H}$, whilst *Spartina anglica*, *Limonium vulgare* and *Salicornia europaea* were typically among the most ^2H -enriched. The exception to this overall pattern among species occurred in March, when all species were characterized by $\delta^2\text{H}$ values between -26‰ and -20‰ (Fig. 5; Supplementary Information Table 4). These extremely negative leaf water $\delta^2\text{H}$ profiles were significantly different (Minitab v.16, 2013, student's t-test, $P > 0.05$, $n = 10$ individuals per sampling interval comparing those species growing from March to September 2012) to those observed for the same species in all other sampling intervals during 2012.

Leaf water samples collected at 7:30-8:00 and 12:00-14:00 from *Elytrigia atherica*, *Atriplex portulacoides* and *Suaeda vera* at the ridge site allowed us to investigate diurnal shifts in leaf water isotopic composition. The C₃ grass *Elytrigia atherica* showed the greatest shift in the $\delta^2\text{H}$ of leaf water: it was 19‰ more positive at 12:00-14:00 than at 7:30-8:00. Leaf waters from two other plants showed a ^2H -enrichment of only 5-6‰ (Fig. 6; Supplementary Information Table 5).

Statistical analysis (Minitab v.16, 2013) of interspecies variation in leaf water isotopic composition at each sampling interval indicated that leaf water $^2\text{H}/^1\text{H}$ was not significantly different (Mann-Whitney U test, $P>0.05$, $n=8$ for comparison of species growing from March to September 2012; $n=6$ for comparison of species growing from May to September 2012) among the Stiffkey species. However, *Phragmites australis*, the species that generally had the most ^2H -depleted leaf water isotopic signatures, was an exception. Leaf water from *Phragmites* was significantly different from the C₄ grass *Spartina anglica*, and the C₃ species *Salicornia europaea*, *Limonium vulgare*, and *Atriplex portulacoides* (student's t-test, $P<0.05$, $n=6$ individuals per species), but could not be distinguished statistically from leaf water from the other C₃ monocot *Elytrigia atherica*.

4.4 *n*-Alkane $^2\text{H}/^1\text{H}$ composition

Analysis of molecular distributions of *n*-alkanes from the sampled species (Supplementary Information Table 1 and 2) showed that *n*-C₂₇ and *n*-C₂₉ alkanes were the most abundant across all species. Because *n*-C₂₇ and *n*-C₂₉ alkane $\delta^2\text{H}$ values were strongly correlated across the growing season (Fig. 2 in the Supplementary Information), we focused only on *n*-C₂₉ $\delta^2\text{H}$ values in all subsequent data analysis. The mean *n*-C₂₉ $\delta^2\text{H}$ values from June 2011 showed a

total interspecies variation of 98‰, with the C₃ grass *Elytrigia atherica* having the most ²H-depleted *n*-C₂₉ value and *Suaeda vera* the most ²H-enriched. Species collected from multiple sampling sites showed very limited micro-habitat dependent variation ranging from 1‰ (i.e. below the observed maximum intraspecies variability of 6‰ in *n*-C₂₉ ²H/¹H) (*Suaeda vera*) to 9‰ (*Atriplex portulacoides*). The greatest interspecies range in δ²H_{*n*-C₂₉} was observed at the ridge site (93‰), while the lowest occurred in the upper marsh (24‰). *n*-C₂₉ from C₃ grasses was on average 45‰ more ²H-depleted than that from the C₄ *Spartina anglica*. Overall, we observed the following pattern for *n*-C₂₉ alkane δ²H values: succulents > perennial herbs > evergreen shrubs > C₄ grass > C₃ monocots (Fig. 4; Supplementary Information Table 3).

The mean δ²H values of *n*-C₂₉ alkane from the 2012 growing season were remarkably consistent for each individual species across all the sampling intervals (Fig. 5; Supplementary Information Table 4). Seasonal variation from March – September was the highest in the evergreen succulent *Suaeda vera* (44‰), and the lowest in the annual succulent *Salicornia europaea* (5‰). For all other species, seasonal variation in their leaf wax ²H/¹H composition fell within the range of 10-35‰. Statistical analysis (Minitab v.16, 2013) confirmed that these differences are not significant (Mann-Whitney U test, P>0.05, n=10 for March 2012; n=14 for May, August and September 2012).

The greatest interspecies variation in *n*-C₂₉ occurred in August (120‰), however variability among species exceeded 100‰ for all 2012 study intervals. *Elytrigia atherica* and *Phragmites australis* consistently recorded the most negative δ²H values. However, unlike in the leaf water – where *Phragmites* was generally more negative than *Elytrigia* – the *n*-C₂₉ alkane δ²H values of *Elytrigia* were between 23 and 54‰ more negative than those of *Phragmites* across the entire growing season. In addition, the most ²H-enriched *n*-C₂₉ values

were observed in *Suaeda vera*, *Limonium vulgare* and *Salicornia europaea*, with *Spartina anglica* – a species with one of the more positive leaf water $\delta^2\text{H}$ values – having intermediate $n\text{-C}_{29}$ alkane $\delta^2\text{H}$ values across all sampling intervals (Fig. 5). Cross-plotting the $n\text{-C}_{29}$ alkane $\delta^2\text{H}$ data and ACL values (Fig. 1 in the Supplementary Information) for September 2012 did not show any correlation between these two parameters (Fig. 3 in the Supplementary Information).

Statistical analysis of interspecies variation in leaf wax hydrogen isotope compositions among all sampled species across the study period (Minitab v.16, 2013) revealed that the $^2\text{H}/^1\text{H}$ values of waxes were significantly different among most species (Mann-Whitney U test, $P < 0.05$, $n = 8$ for species growing from March to September 2012; $n = 6$ for species growing from May to September 2012). Notable exceptions include a) *Suaeda vera*, and *Limonium vulgare*, and b) the two succulents *Suaeda vera* and *Salicornia europaea*.

4.5 $^2\text{H}/^1\text{H}$ fractionation between soil, xylem and leaf water and $n\text{-C}_{29}$ alkane

Halophyte species are exceptions to the rule that plants do not fractionate environmental water during root uptake (Waisel, 1972; Ellsworth and Williams, 2007). ^2H -discrimination occurring during water uptake among the Stiffkey halophytic species was calculated using the approach of Ellsworth and Williams (2007): $\Delta^2\text{H} = \delta^2\text{H}_{\text{soil water}} - \delta^2\text{H}_{\text{xylem water}}$.

$\Delta^2\text{H}$ was the highest in the evergreen species *Atriplex portulacoides* and *Suaeda vera* for all halophyte species at Stiffkey (28‰) and the lowest in *Limonium vulgare* (4‰). The C_4 grass *Spartina anglica* had a $\Delta^2\text{H}$ value of 13‰. The values reported here exceed those of Ellsworth and Williams (2007), who only reported data from woody xerophytes.

Epsilon values were calculated to approximate $^2\text{H}/^1\text{H}$ fractionation between mean $n\text{-C}_{29}$ $\delta^2\text{H}$ values and soil water ($\epsilon_{\text{wax/sw}}$), xylem water ($\epsilon_{\text{wax/xw}}$), and leaf water ($\epsilon_{\text{wax/lw}}$) using the following equation:

$$\epsilon_{\text{wax/water}} = \frac{\left(\frac{^2\text{H}}{^1\text{H}} \right)_{\text{wax}}}{\left(\frac{^2\text{H}}{^1\text{H}} \right)_{\text{water}}} - 1 = \frac{(\delta^2\text{H})_{\text{wax}} + 1}{(\delta^2\text{H})_{\text{water}} + 1} - 1$$

where $\delta^2\text{H}_{\text{water}}$ represents the hydrogen isotope composition of the leaf water or soil water as appropriate. Epsilon and delta values are reported in per mil (‰), and therefore this equation implies multiplication by 1000 (Cohen et al., 2007).

In June 2011, the total variation in ϵ between $n\text{-C}_{29}$ and leaf water exceeded 100‰ (Fig. 7a & 7b). Similar differences were identified throughout the 2012 growing season when the total variation in $\epsilon_{\text{wax/lw}}$ exceeded 86‰ for all sampling intervals (Fig. 8). The greatest range in $\epsilon_{\text{wax/lw}}$ during the growing season was observed in August (109‰), and the lowest in September (86‰). The C_3 grass *Elytrigia atherica* consistently had the lowest $\epsilon_{\text{wax/lw}}$ value (-184 to -229‰), whilst *Suaeda vera* and *Limonium vulgare* recorded the highest (-79 to -144‰). Across all species, there was a general trend for $\epsilon_{\text{wax/lw}}$ to become lower as the growing season progresses (Fig. 8; Supplementary Information Table 4). The variation in fractionation factors calculated for the plant species at Stiffkey is the largest range in $\epsilon_{\text{wax/lw}}$ reported to date for saltmarsh environments (c.f. Romero and Feakins, 2011). $\epsilon_{\text{wax/sw}}$ values

for species growing at the three sites in 2012 ranged from -64‰ for *Salicornia* in March to -228‰ for *Elytrigia atherica* in September. $\epsilon_{\text{wax/sw}}$ variability among the different plant species exceeded 89‰ throughout the growing season (Supplementary Information Table 4).

The C₄ grass, *Spartina anglica*, has $\epsilon_{\text{wax/lw}}$ values that are higher (by up to 74 ‰) than those observed for the C₃ grass *Elytrigia atherica*. When the *Spartina* data are compared with other C₃ species collected in March and May 2012, $\epsilon_{\text{wax/lw}}$ for *Spartina* is only 5-6‰ higher than in *Atriplex*, although it is 15-36‰ lower than the apparent fractionation observed in *Suaeda* and *Limonium*. As the growth season progresses, the difference in $\epsilon_{\text{wax/lw}}$ among these species increases: in August, where the maximum variation is observed, the ²H/¹H fractionation between leaf water and leaf wax *n*-C₂₉ in *Spartina* is between 25 and 53‰ lower than these other C₃ shrubs and herbs (Supplementary Information Table 2).

5. DISCUSSION

Many previous studies have sought to explain variation in *n*-alkane ²H/¹H composition among different plant species by reference to the physical processes that control the movement of water molecules inside, outside and within the leaf. If we therefore, assume that interspecies variation in our leaf wax lipid $\delta^2\text{H}$ is primarily driven by differences in the isotopic composition of leaf water, it follows that the > 100‰ range in *n*-alkane ²H/¹H compositions observed should be accounted for by a series of scenarios which affect leaf water $\delta^2\text{H}$. These mechanisms include: (i) differences in the isotopic composition of soil water among the three marsh sub-environments; (ii) differences in the isotopic composition of soil water throughout the growing season; (iii) interspecies differences in the isotopic

composition of xylem water, reflecting root uptake of soil water and transport to the leaf, and; (iv) interspecies differences in the isotopic composition of leaf water among plant life forms due to differences in leaf structure, affecting the transpiration of water within the leaf. Each of these scenarios will be considered below, to assess whether they can account for the variation observed in $\delta^2\text{H}_{n\text{-C}29}$ among the Stiffkey plants.

5.1 The significance of spatial differences in soil water

Salt marshes are of great significance in lowland coastal regions (Allen, 2000) and represent important depositional environments because they are divisible into discrete micro-environmental zones based on topography and tidal inundation (Vince and Snow, 1984). This characteristic makes salt marshes ideal for studying plant/environment interactions (Vince and Snow, 1984; Romero and Feakins, 2011). Soils and sediments at Stiffkey receive water inputs from two sources: Sea water, which inundates the lower marsh and low-lying areas of the upper marsh daily, and meteoric precipitation, which is especially important on the ridge where no tidal inundation occurs.

Previous studies (Romero and Feakins, 2011) show that environmental water varies in isotopic composition across salt marsh sites. Our data from 2012 demonstrates this (Supplementary Information Table 4), showing that the LM and UM (both sites that regularly receive inputs of saline water) have relatively similar isotopic compositions of source water (-2‰ to +2‰ between May and September 2012). Soil water from the ridge at Stiffkey is up to 35‰ more ^2H -depleted than the other two sampling sites.

552 Despite these spatial changes in the isotopic composition of environmental water, large
553 variations in the $\epsilon_{\text{wax/lw}}$ values observed within each sub-environment at Stiffkey (LM, R,
554 UM) in June 2011 (Fig. 7a) suggest that source water isotopic composition is not a major
555 factor controlling the hydrogen isotope signals preserved in the $n\text{-C}_{29}$ alkane. This is
556 supported by the limited variation observed in leaf water and n -alkane samples from selected
557 plant species sampled in June 2011. Although no soil waters were collected in June, sampling
558 of species growing in more than one location at Stiffkey allows for evaluation of the impact
559 of marsh sub-environment on the $^2\text{H}/^1\text{H}$ composition of leaf waters and leaf wax lipids. In
560 theory, if spatial variation in environmental water across these sub-environments is
561 significant, we would expect samples of the same individual species from multiple sites to
562 have different $\delta^2\text{H}$ leaf water and n -alkane compositions. Minor discrepancies in leaf water
563 are observed in each species depending upon the particular sub-environment, for example
564 13‰ between *Atriplex portulacoides* at the R and UM sites and 10‰ between *Triglochin*
565 *maritima* at the LM and UM sites; Fig. 4). However, the magnitude of this spatial variability
566 is insignificant when compared with the range of interspecies $\delta^2\text{H}_{\text{lw}}$ values observed across
567 the marsh as a whole (29‰). Differences in mean $\delta^2\text{H}_{n\text{-C}_{29}}$ values for these species also show
568 insignificant variation depending on sampling site – *Limonium*, *Triglochin* and *Suaeda* all
569 vary by less than 5‰ between the LM and UM, while *Atriplex* shifts isotopically by 12‰
570 (Fig. 4; SI Table 3). Again, the magnitude of these site-specific isotopic differences in
571 individual species is negligible when compared with the ~100‰ interspecies variation in
572 $\delta^2\text{H}_{n\text{-C}_{29}}$ among all sampled plants. In addition, $\epsilon_{\text{wax/lw}}$ values from *Suaeda*, and *Limonium*
573 show remarkable consistency across multiple sampling sites, with the maximum site-specific
574 variation in one species (10‰ in *Triglochin*; 11‰ in *Atriplex*) an order of magnitude less
575 than the total range in $\epsilon_{\text{wax/lw}}$ observed in the data set as a whole (Fig. 7a, and 7b). We

conclude, therefore, that differences in the isotopic composition of soil water among site sub-environments cannot explain interspecies variation in leaf water or *n*-alkane $^2\text{H}/^1\text{H}$ composition.

5.2 The significance of temporal differences in soil water

In order to examine the influence of environmental water fully, it is important to consider whether differences in plant growth strategy expose them to seasonal variation in the source water $\delta^2\text{H}$ signal. There is conflict in previous research over whether the *n*-alkane $^2\text{H}/^1\text{H}$ is “locked in” at the beginning of the growing season or continually shifts in response to environmental or biological stimuli. Sachse et al. (2010) concluded that the *n*-alkane $\delta^2\text{H}$ values for field-grown barley were fixed early during the growing season and did not show seasonal shifts as the plants matured. A similar conclusion was reached by Tipple et al. (2013), who analysed the $^2\text{H}/^1\text{H}$ composition of *n*-alkanes, stem water, and leaf water from the riparian angiosperm *Populus angustifolia* throughout a growing season. Leaf water values showed considerable seasonal variation of 55‰, however, *n*-alkane $\delta^2\text{H}$ values remained relatively consistent in the mature leaf. This was interpreted to reflect the fixing of the *n*-alkane $\delta^2\text{H}$ signal during the bud break period, where new waxes are produced from water and stored sugars, suggesting that the *n*-alkane $^2\text{H}/^1\text{H}$ composition reflected these mixed biosynthate sources rather than providing an integrated signal of the growing season as a whole. In contrast, other studies propose that leaf waxes turnover continuously. Jetter and Schäffer (2006) considered that wax production was dynamic, with turnover and recycling of dominant compound classes during leaf development, whilst Gao et al. (2012) quantified regeneration rates of leaf wax compounds by the application of labelled irrigation water and concluded that *n*-C₂₇ – *n*-C₃₁ *n*-alkanes are replaced over a timescale of 71-128 days.

601

602 Plant species growing at our study site are regularly exposed to strong winds from the North
603 Sea, in combination with rain, and tidal inundation. These environmental factors are likely to
604 abrade waxes from the surface of leaves, which means plants have to produce further wax to
605 maintain their protective coating (Shepherd and Griffiths, 2006; Kahmen et. al., 2013). Given
606 their exposed coastal location, it is likely that plants growing at Stiffkey were regularly
607 required to replenish their leaf waxes throughout the growing season. On that basis, we
608 hypothesise that if plants at Stiffkey were synthesising their leaf waxes at different times of
609 year, they may be utilising soil water with different $^2\text{H}/^1\text{H}$ compositions. We therefore, tested
610 whether any temporal variation in soil water isotopic composition (-36‰ in March, +2‰ in
611 May 2012) could adequately account for the interspecies variation in leaf wax $\delta^2\text{H}$ we
612 observed in our data.

613

614 Plants at Stiffkey are known to have varied growth strategies. *Suaeda vera*, for example, is an
615 evergreen succulent (Schirmer and Breckle, 1982), *Atriplex portulacoides* is an evergreen
616 shrub (Corerria das Neves et al., 2008), whilst *Limonium vulgare* (Boorman, 1967), *Spartina*
617 *anglica* and *Phragmites australis* (Burke et al., 2000) are all perennials (the latter two species
618 are grasses, while the former is a flowering perennial). In addition to our soil water data,
619 mean monthly interpolated $\delta^2\text{H}$ profiles of meteoric water at Stiffkey, obtained using the
620 Online Isotopes in Precipitation Calculator (OIPC), version 2.2 (Bowen et al., 2005), were
621 also used for consideration of this temporal parameter (Supplementary Information Table 6).

622

623 In order to evaluate the importance of temporal changes in soil water isotope composition, it
624 is first necessary to consider sources of water inputs at the marsh. At the LM and UM sites,
625 seawater is the main source and is assumed to have an invariant isotopic value throughout the

year (see for example Sessions, 2006). At Site 3 seawater ingress is through a dendritic network of tidal channels (Figure 1), and the proliferation of *Triglochin maritima* and *Salicornia europaea*, species known to require saline water, attest to the importance of seawater inputs to the upper marsh (Allison, 1992; Davy and Bishop, 1991). However, early in the growing season, March soil water $\delta^2\text{H}$ from the lower marsh shows a considerably more ^2H -depleted value than for other sampling intervals. Examination of local weather station monitoring data (MIDAS, UK Meteorological Office) shows that on the day of sampling rainfall occurred at the site before sampling and after the last high tide. The estimated value for $\delta^2\text{H}$ of precipitation in North Norfolk in March is c. -62‰ (OIPC), and assuming a seawater $\delta^2\text{H}$ value of 0‰, we calculate that rainfall contributed ~40% of the $^2\text{H}/^1\text{H}$ soil water signal in this sample. It is likely, however, that with the next high tide, the importance of this meteoric water input would be negated. The $\delta^2\text{H}$ data from May and September 2012 support this, as they have a ‘near-seawater’ isotopic signature, ranging from -2 to +2‰ (SI Table 4). Therefore, regardless of the season during which LM and UM plant species synthesised leaf waxes, temporal isotopic shifts in soil water cannot explain interspecies variation in the *n*-C₂₉ alkane $\delta^2\text{H}$ values observed in these two locations.

In contrast, the ridge is only rarely inundated by tides and is dominated by meteoric precipitation, which explains why our most ^2H -depleted soil water is found at this site (SI Table 4). Examination of mean monthly interpolated $\delta^2\text{H}$ values of meteoric water at the Stiffkey site (OIPC; Supplementary Information Table 6) for our sampling periods show, however, modelled precipitation $^2\text{H}/^1\text{H}$ ranges from -62‰ (March) to -48‰ (September). Soil waters from the ridge are consistently more ^2H -enriched than these meteoric precipitation $\delta^2\text{H}$ profiles, which we attribute to two likely causes. Firstly, as daytime temperatures rise during the growing season, soil evaporation will increase, particularly from

the near-surface depths sampled, resulting in increasing ^2H -enrichment in the remaining pore water. Secondly, as the water table at the site is relatively high, an upwards movement of water through soil capillaries (“capillary rise”, Plaster, 2009), particularly during warmer summer months, may carry ^2H -enriched seawater towards the soil surface (Plaster, 2009). When we consider these temporal shifts in environmental water $^2\text{H}/^1\text{H}$ composition in the context of the interspecies variability in leaf wax *n*-alkane hydrogen isotope compositions observed at this particular sampling site, it is clear that temporal variation in the isotopic composition of soil water and precipitation cannot explain the $\delta^2\text{H}_{n\text{-C}29}$ range among the ridge species. In our study, soil water $\delta^2\text{H}$ varied by 31‰ at the ridge across the 2012 growth season, while the average interspecies range in $\delta^2\text{H}_{n\text{-C}29}$ consistently exceeded 100‰.

In addition to consideration of seasonal shifts in the isotopic composition of environmental water, soil samples collected from the ridge between 7:30 and 8:00 on the 7th of September 2012 allowed us to investigate diurnal changes in soil water $\delta^2\text{H}$. Sachse et al. (2010) suggested that one reason a direct 1:1 relationship was not observed between the $\delta^2\text{H}$ of midday leaf water and $\delta^2\text{H}_{n\text{-C}29}$ in barley was that plants were synthesising these compounds from water that had not been subjected to diurnal ^2H -enrichment. In our study, the hydrogen isotope signature of soil water from the ridge between 7:30 and 8:00 was 16‰ lower compared with soil samples collected between 12.00 and 14.00 (SI Table 5), while leaf waxes from species sampled at the ridge in September varied by ~90‰. Therefore, diurnal variation in environmental water also cannot explain the range in interspecies $\delta^2\text{H}_{n\text{-C}29}$ observed in the coastal plants at Stiffkey.

5.3 The significance of soil water uptake by halophytes and non-halophytes

Sachse et al. (2010) considered the possibility of a ^2H -depleted pool of water occurring in plants as a source of hydrogen for lipid synthesis, whereas McInerney et al. (2011) suggested that xylem water could be used by the plant in preference to leaf waters for lipid biosynthesis. Xerophytes and halophytes are exceptions to the general rule that isotopic fractionation does not occur during water uptake by plants (Ellsworth and Williams, 2007). In these drought and salinity tolerant plants, the mechanism of water uptake by roots is via the symplastic pathway, requiring transport from cell to cell. This transport from cytoplasm of one cell to cytoplasm of the next cell requires energy, and hence leads to diffusional $^2\text{H}/^1\text{H}$ fractionation of water molecules, with xylem waters becoming ^2H -depleted relative to environmental water (Ellsworth and Williams, 2007).

Xylem waters collected between 12:00 and 14:00 at Stiffkey on the 7th of September 2012 allow us to consider whether interspecies variation in fractionation occurring during water uptake ($\Delta^2\text{H}$) can explain the variation in $\delta^2\text{H}_{n-\text{C}29}$ in our data set. $\Delta^2\text{H}$ values for the Stiffkey halophytes (those species with an Ellenberg value in excess of 4) show a much greater range than that published by Ellsworth and Williams (2007); however the maximum fractionation observed for *Atriplex* is still only 28‰, compared with a minimum fractionation of 4‰ in *Limonium vulgare*. This variation in fractionation during water uptake does not explain the 41‰ difference between their $\delta^2\text{H}_{n-\text{C}29}$ values. Equally, *Atriplex* and *Suaeda* growing on the ridge have the same $\Delta^2\text{H}$ values (28‰), but their $\delta^2\text{H}_{n-\text{C}29}$ values differ by 25‰

Some species at Stiffkey are merely salt tolerant and not classified as true halophytes. These include the common reed *Phragmites australis* (Hill et al., 1999; Mauchamp and Mésleard, 2001) and *Elytrigia atherica* (Hill et al., 1999). Interestingly, these species also show xylem water values more negative than the soil water at their sampling location at the ridge site (Fig.

2). Because these plants are not true halophytes, it is unlikely that this is due to their utilisation of the symplastic pathway. Rather, we suggest this phenomenon arises from these species having rooting depths below that sampled for soil water, i.e. deeper than *c.* 10 cm. This would allow them to take up water that has not been subjected to evaporative ^2H -enrichment. *Phragmites australis* in particular has been known to develop roots as deep as 3 m (Thevs et al, 2007), which would allow it to exploit groundwater below the sampling range of this study.

5.4 The significance of leaf water

Physical differences among plants with different life forms, leading to various patterns of utilization of environmental water, have been used to explain variation in $\delta^2\text{H}$ *n*-alkane values observed between both woody plants and grass (Liu et al., 2006). For instance, morphological characteristics have been identified as factors exerting a strong influence upon leaf water isotopic ^{18}O -enrichment (Helliker and Ehleringer, 2002; Barbour et al., 2004). Kahmen *et al.* (2008) suggested that leaf water isotopic ^{18}O -enrichment can differ even among species that are closely related because of differences in the “effective path length” (the distance that water is required to flow from source to evaporation site) in their leaves, which would influence the flow of isotopically enriched water back from the sub-stomatal cavity. Similar factors could potentially influence hydrogen isotopic composition of leaf water as well.

Studies seeking to apply factors relating to leaf water ^2H -enrichment to *n*-alkane data have attempted to explain observed variation in *n*-alkane $^2\text{H}/^1\text{H}$ in terms of differences in plant life form on the basis that these physical differences could have influenced evapotranspiration of the source water used by the plant during biosynthesis (Liu et al., 2006). At Stiffkey, plants

display very different life forms ranging from succulents, grasses and shrubs. However, leaf waters extracted from morphologically distinct species at the same site in June 2011 (Fig. 4) show very little variation in their $\delta^2\text{H}$ values. For example, the ridge contains a range of plant species that differ significantly with respect to their leaf morphology. The reed *Phragmites australis* has large, elongated leaves up to 30 cm long and 2 cm wide, while the leaf succulent *Suaeda vera* has leaves that are only 3 mm in length and approximately 1.5 mm in diameter. However, the $\delta^2\text{H}_{\text{lw}}$ values range from +5‰ to +21‰ whilst $\delta^2\text{H}_{n\text{-C}_{29}}$ values differ by over 65‰ between these species. Similar patterns can be found in the seasonal data from 2012, where statistical analysis (Mann-Whitney U test, $P > 0.05$, $n = 8$ for comparison of species growing from March to September 2012; $n = 6$ for comparison of species growing from May to September 2012) confirms that interspecies variation in leaf water hydrogen isotope composition is generally not significant. Even if we compare species with extreme variation in leaf morphology such as *Phragmites australis* and *Suaeda vera* – where a statistically significant difference in leaf water does exist – leaf water $^2\text{H}/^1\text{H}$ between these two plants only ranges from 6 to 12‰ between May and September 2012. Leaf wax $n\text{-C}_{29}$ $^2\text{H}/^1\text{H}$ values, however, differ consistently by over 50‰ during the same period (Fig. 5).

When all the species sampled at Stiffkey are considered, variability in leaf water $\delta^2\text{H}$ composition is three times lower than that observed in $\delta^2\text{H}_{n\text{-C}_{29}}$ in June 2011, and consistently 4-5 times lower throughout the seasonal time series from 2012. $^2\text{H}/^1\text{H}$ composition of n -alkanes ($\delta^2\text{H}_{n\text{-C}_{29}}$) varies across all seasonal sampling periods at Stiffkey by over 100‰, with the greatest variability observed in August (120‰). In contrast, leaf waters across the same period ($\delta^2\text{H}_{\text{lw}}$) show a total variation of only 29‰ (Supplementary Information Table 4). This contrast between a large variability of n -alkane $\delta^2\text{H}$ and a small range of leaf water $\delta^2\text{H}$ values is particularly striking at the beginning and mid stages of the growth season. In March

2012, the mean values of n -C₂₉ alkane show 103‰ variation among sampled species, with only 6‰ shifts in leaf water, whilst in August 2012 the n -C₂₉ range exceeds 120‰ and leaf waters vary by only 29‰. *Phragmites australis* generally has the most negative leaf water ²H/¹H profile, whilst *Limonium vulgare*, *Spartina anglica* and *Salicornia* have leaf waters that are all generally ²H-enriched compared with other species. Statistical analysis (student's t-test, $P > 0.05$, $n = 10$ individuals per sampling interval comparing those species growing from March to September 2012) of seasonal shifts in leaf water ²H/¹H among each species shows that March 2012 is significantly different from all other months. The range in leaf water $\delta^2\text{H}$ in March 2012 is quite limited compared with all other sampling periods. Even if the n -alkane ²H/¹H profiles of our sampled species are in fact fixed at the time of leaf expansion, e.g. as suggested by Tipple et al. (2013), the range in $\delta^2\text{H}_{n\text{-C}_{29}}$ alkanes observed in March 2012 (103‰) have therefore to be attributed to something other than leaf water isotopic composition.

In addition, our data also show that ²H-depletion and ²H-enrichment in leaf water and n -C₂₉ alkane values do not co-vary, i.e. any similarity in leaf water ²H/¹H composition does not necessarily lead to a similarity in n -alkane $\delta^2\text{H}$ values. Figure 2 presents data from the September 2012 sampling period, and shows that for species with very similar leaf water ²H/¹H compositions, n -alkane values can vary considerably. For example, whilst *Limonium vulgare* and *Salicornia* have the most ²H-enriched leaf water and n -alkane values, *Atriplex portulacoides*, *Suaeda vera* and *Elytrigia atherica* have leaf water values within 8‰ of each other whereas their n -alkane values vary by up to 89‰. In addition, the difference between $\delta^2\text{H}_{\text{lw}}$ of *Limonium* and *Elytrigia* is 19‰, while the range in n -C₂₉ between these species $\delta^2\text{H}$ reaches 105‰.

Similar discrepancies between the magnitude of differences in the hydrogen isotope composition of leaf waters and the hydrogen isotope composition of the n -C₂₉ alkane are found throughout all the sampling periods. For example, data collected in June 2011 (Fig. 4; Supplementary Information Table 2) *Triglochin maritima* from the low marsh has the most ²H-depleted leaf water value (+22‰) of plants found in this sub-environment, but this does not result in *Triglochin maritima* having the most ²H-depleted n -C₂₉ alkane value. Similarly, the C₄ grass *Spartina anglica* has the most ²H-depleted n -C₂₉ alkane (-156‰) value in the low marsh, but one of the more ²H-enriched leaf waters (+27‰). This lack of correlation between leaf water and leaf wax $\delta^2\text{H}$ at the plant species level is also apparent in the June 2011 dataset when species having very similar leaf water values – *Limonium vulgare* and *Salicornia europaea* differ by only 1‰ in the low marsh – synthesized n -C₂₉ alkanes that differ by as much as 20‰ (Fig. 4).

At the ridge, where the greatest range in $\epsilon_{\text{wax/lw}}$ values is observed in June 2011, this lack of correlation between leaf water and n -C₂₉ alkane ²H/¹H composition is also present (Fig. 4). Here, it is the C₃ reed, *Phragmites australis* that has the most ²H-depleted leaf water (+5‰), but the n -C₂₉ n -alkane $\delta^2\text{H}$ value for this species does not follow this trend (Fig. 4). The most ²H-depleted n -C₂₉ alkane value on the ridge is in fact found in another C₃ grass, *Elytrigia atherica*, which has a leaf water $\delta^2\text{H}$ value of +15‰. As observed in the low marsh, similar leaf water δD values do not result in similar n -C₂₉ alkane $\delta^2\text{H}$ values: *Atriplex portulacoides*, and *Suaeda vera* and *Elytrigia atherica* all record leaf water ²H/¹H values ranging from +15 to +21‰, but differ by 93‰ in terms of their n -C₂₉ alkane $\delta^2\text{H}$ values. Even in the upper marsh, where the $\delta^2\text{H}$ values display the smallest overall range among plant species, *Triglochin maritima* and *Atriplex portulacoides* record the highest leaf water $\delta^2\text{H}$ values but in contrast have lowest n -C₂₉ alkane $\delta^2\text{H}$ values (Fig 4). Statistical analysis of interspecies

variation in n -C₂₉ hydrogen isotope composition supports our finding that leaf water $^2\text{H}/^1\text{H}$ is of limited relative importance in controlling leaf wax $\delta^2\text{H}$ values. Variation in midday leaf water $\delta^2\text{H}$ among the sampled species was not found to be statistically significant, while in contrast interspecies variation in n -C₂₉ $\delta^2\text{H}$ was, suggesting some other mechanism was responsible for the >100‰ range in n -C₂₉ we report.

Previous research has suggested that some plants may utilise pre-dawn leaf water that has not been subject to diurnal evaporative enrichment when synthesising leaf wax n -alkanes (Sachse et al., 2010). Leaf water samples collected between 7:30 and 8:00 from three species capturing the full range of n -C₂₉ alkane $\delta^2\text{H}$ values at the ridge site (*Elytrigia atherica*, *Atriplex portulacoides* and *Suaeda vera*) show a maximum variation of 25‰ (Fig. 6). However, it is insufficient to explain the 89‰ range in the n -C₂₉ alkane $\delta^2\text{H}$ values from these species. Taken in consideration with the xylem water discussed above, it becomes apparent that even in the case of the most extreme theoretical scenario whereby *Elytrigia atherica* – the species with the lowest $^2\text{H}/^1\text{H}$ n -C₂₉ value – made use of early morning xylem water (-47‰) for lipid synthesis, while *Suaeda vera* (the species with the highest $^2\text{H}/^1\text{H}$ n -C₂₉ value) instead used evaporatively ^2H -enriched midday leaf water (+4‰), the maximum range in the pools of water for lipid synthesis would be 51‰ which still does not satisfactorily explain the 89‰ difference in $\delta^2\text{H}_{n\text{-C}_{29}}$ between them.

5.5 Comparison of $^2\text{H}/^1\text{H}$ fractionation among C₃ and C₄ plants at Stiffkey with previously published research

Earlier work has suggested that C₃ vs. C₄ plants have relatively invariant fractionation factors between n -alkanes and leaf/source water. Examples include the generalised apparent

fractionation factors between leaf water and *n*-alkyl lipids calculated for C₃ (-117±27‰) and C₄ (-132±12‰) plants (Chikaraishi and Naraoka, 2003; Chikaraishi et al., 2004), which continue to be applied to modern vegetation studies (Tippie et al., 2013) and palaeoclimate reconstructions (van Soelen et al., 2013; Lieder et al., 2013). Our data suggest these predicted values may not reflect the true extent of plant lipid ²H/¹H diversity - if, for example, fractionation is calculated between leaf water and the *n*-C₂₉ alkane for September 2012, only half of the C₃ plants sampled have ε_{wax/lw} values that fall within the range predicted by Chikaraishi and Naraoka (2003; 2004; Supplementary Information Table 4). The remaining C₃ species, which include *Elytrigia atherica*, *Phragmites australis* and *Atriplex portulacoides*, have ε_{wax/water} values that are 26-83‰ lower than the predicted values. This lack of agreement with estimated values is found throughout our dataset – in June 2011, only two C₃ species conform to the predicted values (Fig. 7b), while between March and August 2012, only *Limonium vulgare*, *Suaeda vera* and *Salicornia europaea* have ε_{wax/lw} values that regularly fall within the predicted -90 to -144‰ range for C₃ species (Chikaraishi and Naraoka, 2003; 2004). With regards to the C₄-plant group, our calculated ε_{wax/lw} values for the C₄ grass *Spartina anglica* for both June 2011 (-178‰) and the 2012 growth season (-115 to -176‰ between March and September) exceed the range of -120 to -144‰ for C₄ species published by Chikaraishi and Naraoka (2003, 2004; Fig. 7b; Fig. 8).

A consistent difference in apparent fractionation among C₃ and C₄ species has also been identified in some studies. For example, Chikaraishi and Naraoka (2003) presented data suggesting that C₄ species had higher apparent fractionation factors compared with C₃ angiosperms and gymnosperms. However, plant functional types were not distinguished in

this study, and large standard deviations for the mean $\epsilon_{\text{wax/w}}$ values ($C_3 = -116 \pm 25\%$, $C_4 = -133 \pm 12\%$) give rise to a degree of overlap in the range of these values. Bi et al. (2005) published data suggesting that in fact C_4 species are typified by n -alkane $^2\text{H}/^1\text{H}$ compositions of $-150.4 \pm 42.6\%$, while that n -alkane $\delta^2\text{H}$ signatures in C_3 species average $-175.7 \pm 29.5\%$. Smith and Freeman (2006) limited their study to C_3 and C_4 grasses, and found that ϵ values were $\sim 20\%$ more negative in C_3 grasses relative to C_4 grasses, resulting in more negative n -alkane $^2\text{H}/^1\text{H}$ compositions in C_3 grasses. Their result for C_3 and C_4 monocots cannot be explained by gross anatomical differences in leaves and, therefore, it has been hypothesised that differences in the interveinal distance among C_3 and C_4 grasses – alongside difference in the extent of the backflow of enriched water from around the stomata – are responsible for the variation (Smith and Freeman, 2006; Tierney et al., 2010).

One implication of such studies is that the considerable scatter in n -alkane $\delta^2\text{H}$ among plants at a specific site is primarily a function of the very negative apparent fractionation between water and leaf wax lipids inherent in C_3 grasses. Our data show that the C_3 grass *Elytrigia atherica* consistently has the largest $\epsilon_{\text{wax/lw}}$ value (up to -227%), followed by the C_3 monocot reed *Phragmites australis* (up to -204%), while the average value for the C_4 *Spartina anglica* in 2012 is $-154 \pm 29\%$. However, the maximum seasonal variability among Stiffkey species, when excluding both C_3 monocots, is still as high as 97% , while for each sampling interval this variability ranges from 30 to 50% (Supplementary Information, Table 4). Similarly, if the C_3 monocots are excluded from consideration in our June 2011 dataset (SI Table 3), the maximum variability excluding *Elytrigia*, *Phragmites* and *Puccinellia maritima* is still 44% . Our data imply that interspecies variation in apparent fractionation in the species at our study site is not explained by differences in C_3 versus C_4 photosynthetic pathways, or indeed in plant life form. The magnitude of variability when C_3 monocots are excluded from

consideration also demonstrates that it may not always be accurate to assume that one plant functional type dictates the magnitude of interspecies variation in *n*-alkane $^2\text{H}/^1\text{H}$ at any given location.

6. CONCLUSION

We have carried out a systematic study of the relationship between the hydrogen isotope composition of soil, xylem and leaf water and the $^2\text{H}/^1\text{H}$ of the *n*-C₂₉ alkane within a range of halophytic and non-halophytic C₃ and C₄ plants growing at Stiffkey marsh in Norfolk, UK. Our data display significant interspecies variation in fractionation between leaf water and leaf wax, ranging from -79 to -229‰ across the 2012 growing season. The > 100‰ range of our $\delta^2\text{H}_{n\text{-C}_{29}}$ data, and the 150‰ range in $\epsilon_{\text{wax/lw}}$ values, extend beyond the typical values for C₃ and C₄ plants put forward in previous studies. We thus infer that reconstruction of palaeohydrological regimes based on estimates such as these may not capture the full complexity of the hydrogen isotope information recorded by these plant groups. The range in our *n*-alkane $\delta^2\text{H}$ cannot be explained by reference to spatial or temporal shifts in the hydrogen isotope composition of soil, xylem or leaf water. We therefore conclude that environmental and physical mechanisms controlling leaf water isotopic composition cannot fully account for the interspecies variation in our *n*-alkane hydrogen isotope data. Instead, our data show that biochemical mechanisms may play a more important role in controlling interspecies variation in (i) *n*-alkane $^2\text{H}/^1\text{H}$ composition, and (ii) fractionation between source water and *n*-alkane $^2\text{H}/^1\text{H}$, than abiotic factors.

Previous research has already identified that biochemical processes may have an important role to play in determining leaf biomarker $^2\text{H}/^1\text{H}$. However little is currently known about

how this mechanism operates in terrestrial plants. We suggest that future studies should make use of an integrated approach and focus on distinguishing biochemically moderated fractionation from environmental and physical factors. The 100‰ range in *n*-alkane $\delta^2\text{H}$ compositions recorded at Stiffkey highlights the fact that any attempt to reconstruct palaeohydrological information from sedimentary leaf-wax lipids needs to fully account for any shifts in $^2\text{H}/^1\text{H}$ composition arising from changes in higher plant assemblages. Further research is necessary to improve our understanding of the relative importance of biosynthetic processes responsible for interspecies variation in leaf-wax lipid $^2\text{H}/^1\text{H}$, because this will determine the nature of the information – environmental signals versus differences in plant biochemistry – recorded in these biomarkers. Only then can the use of *n*-alkane $^2\text{H}/^1\text{H}$ analysis for palaeoclimate reconstructions be fully evaluated.

Acknowledgements

The authors gratefully acknowledge the assistance of Annette Eley, Louise Jones and Joseph Dillon during sample collection, Liz Rix for technical and analytical support during data generation and Professor Anthony Davy who provided valuable insights regarding halophyte plant adaptations in salt marshes. The authors also wish to thank Franz Street and Mike Andrews (University of Reading, UK) for technical and analytical support during the extraction of leaf, soil and stem waters. Professor John Allen (University of Reading, UK), is thanked for the useful discussions regarding salt marsh formation and geomorphology on the north Norfolk coast. We also thank A. Kahmen, S. Feakins and 3 anonymous reviewers for their helpful and constructive comments. This research was funded in part by the University of East Anglia.

923

924

925

REFERENCES

926

927 Allen, J. (2000) Morphodynamics of Holocene salt marshes: a review sketch from the
928 Atlantic and Southern North Sea coasts of Europe. *Quat. Sci. Rev.* **19**, 1155-1231.

929 Allison, S. (1992) The influence of rainfall variability on the species composition of a
930 northern California salt marsh plant assemblage. *Plant Ecol.* **101**, 145-160.

931 Andrews, J., Boomer, I., Bailiff, I., Balson, P., Bristow, C., Chronston, P., Funnell, B.,
932 Harwood, G., Jones, R., Maher, B. (2000) Sedimentary evolution of the north Norfolk
933 barrier coastline in the context of Holocene sea-level change. *Geol. Soc. Spec. Publ.*
934 **166**, 219-251.

935 Barbour, M. M., Schurr, U., Henry, B. K., Wong, S. C., Farquhar, G. D. (2000) Variation in
936 the oxygen isotope ratio of phloem sap sucrose from castor bean. Evidence in support
937 of the Peclet effect. *Plant Physiol.* **123**, 671-680.

938 Barbour, M., Roden, J., Farquhar, G., Ehleringer, J. (2004) Expressing leaf water and
939 cellulose oxygen isotope ratios as enrichment above source water reveals evidence of
940 a Péclet effect. *Oecologia* **138**, 426-435.

941 Bi, X., Sheng, G., Liu, X., Li, C., Fu, J. (2005) Molecular and carbon and hydrogen isotopic
942 composition of *n*-alkanes of plant leaf waxes. *Org. Geochem.* **36**, 1405-1417.

943 Bohnert, H., Jensen, R. (1996) Strategies for engineering water-stress tolerance in plants.
944 *T.I.B. Tech.*, **14**, 89 – 97.

945 Boomer, I and Woodcock, L. (1999) The nature and origin of Stiffkey Meals, North Norfolk
946 coast. *Bulletin of the Geological society of Norfolk*, **49**, 3-13

947 Boorman, L. (1967) *Limonium vulgare* Mill. and *L. humile* Mill. *J. Ecol.* **55**, 221-232.

948 Bowen G. J., Wassenaar L. I., Hobson K. A. (2005) Global application of stable hydrogen
949 and oxygen isotopes to wildlife forensics. *Oecologia* **143**, 337-348.

950 Burke, D., Weis, J., Weis, P. (2000) Release of Metals by the Leaves of the Salt Marsh
951 Grasses *Spartina alterniflora* and *Phragmites australis*. *Estuar. Coast. Shelf S.* **51**,
952 153-159.

953 Callaway, J. C., Delaune, R. D., Patrick, W. H. (1998) Heavy metal chronologies in selected
954 coastal wetlands from Northern Europe. *Mar. Pollut. Bull.* **36**, 82-96.

955 Callaway, J., Delaune, R., Patrick JR, W. (1996) Chernobyl ¹³⁷Cs used to determine sediment
956 accretion rates at selected northern European coastal wetlands. *Limnol. Oceanogr.* **41**,
957 444-450.

958 Chikaraishi, Y., Naraoka, H. (2003) Compound-specific δD and δ¹³C analyses of *n*-alkanes
959 extracted from terrestrial and aquatic plants. *Phytochemistry* **63**, 361-371.

Chikaraishi, Y., Naraoka, H., Poulson, S. (2004) Hydrogen and carbon isotopic fractionations of lipid biosynthesis among terrestrial (C3, C4 and CAM) and aquatic plants. *Phytochemistry* **65**, 1369-1381.

Cohen, E., Cvitaš, T., Frey, J., Homström, B., Kuchitsu, K., Marquardt, R. Mills, I., Pavese, F., Quack, M., Stohner, J., Strauss, H., Takami, M., Thor, A. (Eds) (2007) *Quantities, Units and Symbols in Physical Chemistry*. R. Soc. Chem. Publ. 265 pp. 3rd ed.

Correrria das Neves, J., Ferrerria, L., Vaz, M., Gazarini, L. (2008) Gas exchange in the salt marsh species *Atriplex portulacoides* L. and *Limoniastrum monopetalum* L. in Southern Portugal. *Acta Physiol. Plant.* **30**, 91-97.

Davy, A., Bishop, G. (1991) *Triglochin maritima* L. *J. Ecol.* **72**, 531-555.

Davy, A., Brown, M., Mossman, H., Grant, A. (2011) Colonisation of a newly developing salt marsh: disentangling independent effects of elevation and redox potential on halophytes. *J. Ecol.* **99**, 1350-1357.

Duarte, C.M., Middelburg, J., Caraco, N. (2005). Major role of marine vegetation on the oceanic carbon cycle. *Biogeosciences* **2**, 1–8.

Ellison, A. (1987) Effects of competition, disturbance and herbivory on *Salicornia europaea*. *Ecology* **68**, 576-586.

Ellsworth, P. Z., Williams, D. G. (2007) Hydrogen isotope fractionation during water uptake by woody xerophytes. *Plant Soil* **291**, 93-107.

Farquhar G. D. and Lloyd J. (1993) Carbon and oxygen isotope effects in the exchange of carbon dioxide between terrestrial plants and the atmosphere. In: *Stable Isotopes and Plant Carbon Water Relations*. pp. 47-70, J. R. Ehleringer, A. E. Hall and G. D. Farquhar (Eds). Academic Press.

Farquhar, G., Cernusak, L. (2005) On the isotopic composition of leaf water in the non-steady state. *Funct. Plant Biol.* **32**, 293-303.

Feakins, S. J., Sessions, A. L. (2010) Crassulacean acid metabolism influences D/H ratio of leaf wax in succulent plants. *Org. Geochem.* **41**, 1269 – 1276.

Gao, L., Burnier, A., Huang, Y. (2012) Quantifying instantaneous regeneration rates of plant leaf waxes using stable hydrogen isotope labelling. *Rapid Commun. Mass Spectrom.*, **26**, 115–122

Garcin, Y., Schwab, V. F., Gleixner, G., Kahmen, A., Todou, B., Sene, O., Onana, J. M., Achoundong, G., Sachse, D. (2012) Hydrogen isotope ratios of lacustrine sedimentary *n*-alkanes as proxies of tropical African hydrology: insights from a calibration transect across Cameroon. *Geochim. Cosmochim. Acta* **79**, 106-126.

Helliker, B., Ehleringer, J. (2002). Differential ¹⁸O enrichment of leaf cellulose in C₃ versus C₄ grasses. *Funct. Plant Biol.* **29**, 435-442.

Hilkert, A., Douthitt, C., Schluter, H., Brand, W. 1999. Isotope ratio monitoring gas chromatography/mass spectrometry of D/H by high temperature conversion isotope ratio mass spectrometry. *Rapid. Commun. Mass. Sp.* **13**, 1226-1230.

Hill, M, Mountford, J. , Roy, D., Bunce, R. 1999 *ECOFACT 2a Technical Annex 490 - Ellenberg's indicator values for British Plants*. DETR.

- 1001 Hou, J., D'Andrea, W. J., Macdonald, D., Huang, Y. (2007) Hydrogen isotopic variability in
1002 leaf waxes among terrestrial and aquatic plants around Blood Pond, Massachusetts
1003 (USA). *Org. Geochem.* **38**, 977-984.
- 1004 Huang, Y., Shuman, B., Wang, Y., Webb, T. (2002) Hydrogen isotope ratios of palmitic acid
1005 in lacustrine sediments record late Quaternary climate variations. *Geology* **30**, 1103-
1006 1106.
- 1007 Jeffries, R. (1977) Growth responses of coastal halophytes to inorganic nitrogen. *J. Ecol.* **65**,
1008 847-865.
- 1009 Jeffries, R., Perkins, N. (1977) The effects on the vegetation of the additions of inorganic
1010 nutrients to salt marsh soils at Stiffkey, Norfolk. *J. Ecol.* **65**, 867-882.
- 1011 Jetter, R., Schäffer, S. (2006) Chemical composition of the *Prunus laurocerasus* leaf surface.
1012 Dynamic changes of the epicuticular wax film during leaf development. *Plant*
1013 *Physiol.*, **126**, 1725 – 1737.
- 1014 Kahmen, A., Simonin, K., Tu, K., Merchant, A., Callister, A., Siegwolf, R., Dawson, T.,
1015 Arndt, S. (2008) Effects of environmental parameters, leaf physiological properties and
1016 leaf water relations on leaf water ^{18}O enrichment in different Eucalyptus species,
1017 *Plant Cell Environ.* **31**, 738-751.
- 1018 Kahmen, A., Simonin, K., Tu, K., Goldschmidt, G., Dawson, T. (2009) The influence of
1019 species and growing conditions on the ^{18}O enrichment of leaf water and its impact
1020 on 'effective path length'. *New Phytol.* **184**, 619-630.
- 1021 Kahmen, A., Schefuss, E., Sachse, D. (2013) Leaf water deuterium enrichment shapes leaf
1022 wax *n*-alkane δD values of angiosperm plants I: Experimental evidence and
1023 mechanistic insights. *Geochim. Cosmochim. Acta* **111**, 39-49.
- 1024 Kahmen, A., Hoffman, B., Schefuss, E., Arndt, S., Cernusak, L., West, J., Sachse, D. (2013)
1025 Leaf water deuterium enrichment shapes leaf wax *n*-alkane δD values of angiosperm
1026 plants II: Observational evidence and global implications. *Geochim. Cosmochim.*
1027 *Acta*, **111**, 50 – 63.
- 1028 Leider, A., Hinrichs, K-U., Schefuß, E., Versteegh, G. (2013) Distribution and stable isotopes
1029 of plant wax derived *n*-alkanes in lacustrine, fluvial and marine sediments along an
1030 Eastern Italian transect and their potential to reconstruct the hydrological cycle.
1031 *Geochim. Cosmochim. Acta*, in press.
- 1032 Liu, W., Yang, H., Li, L. (2006) Hydrogen isotopic compositions of *n*-alkanes from terrestrial
1033 plants correlate with their ecological life forms. *Oecologia* **150**, 330-338.
- 1034 Mauchamp, A., Mesléard, F. (2001) Salt tolerance in *Phragmites australis* populations from
1035 coastal Mediterranean marshes. *Aquat. Bot.*, **70**, 39 – 52.
- 1036 McInerney, F. A., Helliker, B. R., Freeman, K. H. (2011) Hydrogen isotope ratios of leaf wax
1037 *n*-alkanes in grasses are insensitive to transpiration. *Geochim. Cosmochim. Acta*, **75**,
1038 541-554.
- 1039 Meier-Augenstein, W. (1999) Applied gas chromatography coupled to isotope ratio mass
1040 spectrometry. *J. Chromatogr. A* **842**, 351-371.
- 1041 Mitsch, W., Gosselink, J. (2000) *Wetlands (3rd Ed)*, John Wiley and Sons.
- 1042 Moeller, I., Spencer, T., French, J. (1996) Wind wave attenuation over saltmarsh surfaces:
1043 preliminary results from Norfolk, England. *J. Coastal Res.* **12**, 1009-1016.

- 1044 Pagani, M., Pedentchouk, N., Huber, M., Sluijs, A., Schouten, S., Brinkhuis, H., Damste, J. S.
1045 S., Dickens, G. R. and the IODP Expedition 302 Expedition Scientists (2006) Arctic
1046 hydrology during global warming at the Palaeocene/Eocene thermal maximum.
1047 *Nature* **442**, 671-675.
- 1048 Pedentchouk, N., Sumner, W., Tipple, B., Pagani, M. (2008) $\delta^{13}\text{C}$ and δD compositions of *n*-
1049 alkanes from modern angiosperms and conifers: An experimental set up in central
1050 Washington State, USA. *Org. Geochem.* **39**, 1066-1071.
- 1051 Plaster, E. (2009) *Soil Science and Management*(5th Ed). Delmar Cengage Learning, pp. 142
1052 – 165.
- 1053 Pye, K. (1992) Saltmarshes on the barrier coastline of north Norfolk, eastern England. In:
1054 Allen, J. R. L & Pye, K. (Eds.) *Saltmarshes: Morphodynamics, Conservation and*
1055 *Engineering Significance*, pp. 148-179, Cambridge University Press.
- 1056 Richardson, C. J. (2000) Freshwater wetlands. In: Barbour, M., Billings, W. (Eds) (2000)
1057 *North American Wetlands*. Cambridge University Press, pp. 449 – 501.
- 1058 Romero, I., C., Feakins, S. J. (2011) Spatial gradients in plant leaf wax D/H across a coastal
1059 salt marsh in southern California. *Org. Geochem.* **42**, 618-629.
- 1060 Sachse, D., Gleixner, G., Wilkes, H., Kahmen, A. (2010) Leaf wax *n*-alkane δD values of
1061 field-grown barley reflect leaf water δD values at the time of leaf formation. *Geochim.*
1062 *Cosmochim. Acta* **74**, 6741-6750.
- 1063 Sachse, D., Radke, J., Gleixner, G. (2004) Hydrogen isotope ratios of recent lacustrine
1064 sedimentary *n*-alkanes record modern climate variability. *Geochim. Cosmochim. Acta*,
1065 **68**, 4877-4889.
- 1066 Sachse, D., Radke, J., Gleixner, G. (2006) δD values of individual *n*-alkanes from terrestrial
1067 plants along a climatic gradient-Implications for the sedimentary biomarker record.
1068 *Org. Geochem.* **37**, 469-483.
- 1069 Sachse, D., Billault, I., Bowen, G., Chikaraishi, Y., Dawson, T., Feakins, S., Freeman, K.,
1070 Magill, C., McInerney, F., van der Meer, M., Polissar, P., Robins, R., Sachs, J.,
1071 Schmidt, H., Sessions, A., White, J., West, J., Kahmen, A. (2012) Molecular
1072 paleohydrology: interpreting the hydrogen-isotopic composition of lipid biomarkers
1073 from photosynthesising organisms. *Annu. Rev. Earth Planet Sci.* **40**, 221-249.
- 1074 Schefuß, E., Schouten, S., Schneider, R. R. (2005) Climatic controls on central African
1075 hydrology during the past 20,000 years. *Nature* **437**, 1003-1006.
- 1076 Schirmer, U., Breckle, S. (1982) The role of bladders for salt removal in some
1077 Chenopodiaceae (mainly *Atriplex* species). In: Sen, D., Rajpurohit, K. (Eds.) (1982)
1078 *Tasks for vegetation science 2: Contributions to the ecology of halophytes*, Dr W Junk
1079 Publishers, pp 215-231.
- 1080 Sessions, A. L. (2006) Seasonal changes in D/H fractionation accompanying lipid
1081 biosynthesis in *Spartina alterniflora*. *Geochim. Cosmochim. Acta* **70**, 2153-2162.
- 1082 Shepherd, T., Griffiths, D. (2006) The effects of stress on plant cuticular waxes. *New Phytol.*,
1083 **171**, 469 – 499.
- 1084 Shu, Y., Feng, X., Posmentier, E. S., Sonder, L. J., Faiia, A. M., Yakir, D. (2008) Isotopic
1085 studies of leaf water. Part 1: A physically based two-dimensional model for pine
1086 needles. *Geochim. Cosmochim. Acta* **72**, 5175-5188.

- 1087 Smith, F., Freeman, K. (2006) Influence of physiology and climate on δD of leaf wax *n*-
1088 alkanes from C₃ and C₄ grasses. **70**, 1172 – 1187.
- 1089 Thevs, N., Zerbe, S., Gahlert, F., Midjiti, M., Succow, M. (2007) Productivity of reed
1090 (*Phragmites australis* Trin. ex Steud.) in continental-arid NW China in relation to
1091 soil, groundwater, and land-use. *J. App. Bot. Food Sci.* **81**, 62-68.
- 1092 Tierney, J., Russell, J., Huang, Y., Sinninghe Damsté, J., Hopmans, E., Cohen, A. (2008)
1093 Northern Hemisphere controls on tropical southeast African climate during the past
1094 60,000 years. *Science* **322**, 252-255.
- 1095 Tierney, J., Russell, J., Huang, Y. (2010) A molecular perspective on Late Quaternary climate
1096 and vegetation change in the Lake Tanganyika basin, East Africa. *Quat. Sci. Rev.*, **29**,
1097 787 – 800
- 1098 Tipple, B., Berke, M., Doman, C., Khachatryan, S., Ehleringer, J. (2013). Leaf-wax *n*-
1099 alkanes record the plant-water environment at leaf flush. *P. Natl. Acad. Sci. USA* **110**,
1100 2659-2664.
- 1101 Tipple, B., Pagani, M. (2013) Environmental control on eastern broadleaf forest species' leaf
1102 wax distributions and D/H ratios. *Geochim. Cosmochim. Acta* **111**, 64-77.
- 1103 UK Meteorological Office. Met Office Integrated Data Archive System (MIDAS) Land and
1104 Marine Surface Stations Data (1853-current), NCAS British Atmospheric Data
1105 Centre, 2012, 10th March 2013; Available from
1106 http://badc.nerc.ac.uk/view/badc.nerc.ac.uk_ATOM_dataent_ukmo-midas
- 1107 van Soelen, E., Wagner-Cremer, F., Sinninghe-Damsté, J., Reichert, G. (2013)
1108 Reconstructing tropical cyclone frequency using hydrogen isotope ratios of
1109 sedimentary *n*-alkanes in northern Queensland, Australia. *Palaeogeogr. Palaeocl.*,
1110 **376**, 66 – 72.
- 1111 Vince, S. W., Snow, A. A. (1984) Plant zonation in an Alaskan salt marsh: I. Distribution,
1112 abundance and environmental factors. *J. Ecol.* **72**, 651-667.
- 1113 Waisel, Y. 1972. *Biology of halophytes*, Academic Press, California.
- 1114 West, A. G., Patrickson, S. J., Ehleringer, J. R. (2006) Water extraction times for plant and
1115 soil materials used in stable isotope analysis. *Rapid Commun. Mass. Sp.* **20**, 1317-
1116 1321.
- 1117 Zhang, Z., Zhao, M., Eglington, G., Lu, H., Huang, C. (2006) Leaf wax lipids as
1118 paleovegetational and paleoenvironmental proxies for the Chinese Loess Plateau over
1119 the last 170 kyr. *Quat. Sci. Rev.* **25**, 575-594.

Figure captions

Figure 1: Aerial photograph (scale *c.* 2.0 x 1.8 km) of Stiffkey marsh, North Norfolk, UK showing the location of the three study sites. Note the presence of an intricate network of inlet channels delivering seawater to low-lying areas adjacent to Site 3 in the upper marsh. (Copyright: Cambridge University Collection of Air Photographs).

Figure 2: Measured soil water $\delta^2\text{H}$ (black diamonds), xylem water $\delta^2\text{H}$ (grey squares), leaf water $\delta^2\text{H}$ (white triangles), and *n*-alkane $\delta^2\text{H}$ (circles) values from all species sampled in September 2012. LV = *Limonium vulgare*, SE = *Salicornia europaea*, SV = *Suaeda vera*, SA = *Spartina anglica*, AP = *Atriplex portulacoides*, PA = *Phragmites australis*, EA = *Elytrigia atherica*. The standard error did not exceed 2‰ for soil, xylem, leaf waters and 9‰ for *n*-alkane measurements.

Figure 3: Measured xylem water $\delta^2\text{H}$ values for three species sampled at the ridge site between 7:30 and 8:00 and again between 12:00 and 14:00 on 7th September 2012. The maximum standard error associated with these measurements was 2‰.

Figure 4: Measured *n*-C₂₉ alkane $\delta^2\text{H}$ (black circles) and leaf water $\delta^2\text{H}$ (white circles) values for all plants sampled across the Stiffkey marsh in June 2011 (“C3” and “C4” refer to plant biochemical pathways). Predicted $\delta^2\text{H}$ values of seawater (grey line) and precipitation (grey shading) are also shown. Plants are grouped by sampling site (Low marsh, Ridge, Upper marsh). Each data point represents a collection of greater than five leaves from a minimum of three separate plants. Maximum standard error associated with these measurements was 5‰ for *n*-alkane values and 1‰ for leaf waters. The isotopic composition of sea water (0‰) is

highlighted by the straight grey line, whilst the grey shaded area illustrates the maximum seasonal range in precipitation $^2\text{H}/^1\text{H}$ composition estimated using the Online Isotopes in Precipitation Calculator (Bowen et al., 2005).

Figure 5: Seasonal variation in $n\text{-C}_{29}$ alkane $\delta^2\text{H}$ and leaf water $\delta^2\text{H}$ values for all plants sampled during the 2012 growth season. Each data point represents a collection of greater than five leaves from a minimum of three separate plants. The maximum standard error associated with these measurements was 8‰ for $n\text{-C}_{29}$ alkane and 2‰ for leaf water.

Figure 6: Measured leaf water $\delta^2\text{H}$ values for three species sampled at the ridge site between 7:30 and 8:00 and again between 12:00 and 14:00 on 7th September 2012. The maximum standard error associated with these measurements was 2‰.

Figure 7: Calculated fractionation ($\epsilon_{\text{wax/lw}}$ ‰) between $n\text{-C}_{29}$ alkane $\delta^2\text{H}$ and leaf water $\delta^2\text{H}$ from samples collected in June 2011 at Stiffkey saltmarsh. Plants are grouped according to a) sampling locations and b) the plant types.

Figure 8: Calculated fractionation ($\epsilon_{\text{wax/lw}}$ ‰) between $n\text{-C}_{29}$ alkane $\delta^2\text{H}$ and leaf water $\delta^2\text{H}$ from samples collected across the 2012 growth season at Stiffkey saltmarsh. SV = *Suaeda vera*, LV = *Limonium vulgare*, SE = *Salicornia europaea*, AP = *Atriplex portulacoides*, SA = *Spartina anglica*, PA = *Phragmites australis*, EA = *Elytrigia atherica*.

SI Figure 1: n -Alkane average chain length (ACL) values from May and September for all species sampled across the 2012 growth season.

1171 **SI Figure 2:** Bivariate plot of ACL and n -C₂₉ alkane $\delta^2\text{H}$ (September 2012) showing no
1172 correlation between the two parameters. Letters in parenthesis denote plant species: AP =
1173 *Atriplex portulacoides*, EA = *Elytrigia atherica*, LV = *Limonium vulgare*, PA = *Phragmites*
1174 *australis*, SE = *Salicornia europaea*, SA = *Spartina anglica*, SV = *Suaeda vera*.

1175

1176 **SI Figure 3:** Bivariate plot of n -C₂₇ and n -C₂₉ alkane $\delta^2\text{H}$ values for all species sampled
1177 across the 2012 growth season showing a strong correlation between the two sets of data.