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

Article

Accepted Version

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

Yvette Eley[*1], Lorna Dawson[2], Stuart Black[3], Julian Andrews[1], and Nikolai Pedentchouk[1]

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

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

[3]Department 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 δ^2H data is complicated by a limited understanding of factors controlling interspecies variation in biomarker $^2H/^1H$ 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 δ^2H 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 δ^2H 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 $^2H/^1H$, however, consistently varied by over 100‰ throughout the 2012 growth season, resulting in an interspecies range in the $\varepsilon_{wax/leaf\ water}$ values of -79 to -227‰. From the discrepancy in the magnitude of these isotopic differences, we conclude that mechanisms driving variation in the $^2H/^1H$ composition of leaf water, including (i) spatial changes in soil water $^2H/^1H$, (ii) temporal changes in soil water $^2H/^1H$, (iii) differences in xylem water $^2H/^1H$, and (iv) differences in leaf water evaporative ^2H-enrichment due to varied plant life forms, cannot explain the range of *n*-alkane δ^2H values we observed. Results from this study suggests that accurate reconstructions of palaeoclimate regimes from sedimentary *n*-alkane δ^2H require further research to constrain those biological mechanisms influencing species-specific differences in $^2H/^1H$ 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 $^{2}H/^{1}H$ composition and that of
46 environmental water. Previous studies have demonstrated a link between the $\delta^{2}H$ 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 $\delta^{2}H$ 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 (Schefuß et al., 2005; Leider et al.,
56 2013) have often implicitly and/or explicitly relied on the assumption that the biosynthetic
57 $^{2}H/^{1}H$ 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 $\delta^{2}H$
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 $\delta^{2}H$ 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 $^{2}H/^{1}H$) and biochemical mechanisms in controlling $^{2}H/^{1}H$ 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 $^{2}H/^{1}H$ measurements were not conducted. Instead, these studies relied on isotopic measurements of environmental water and leaf wax *n*-alkyl compounds, and any differences in $^{2}H/^{1}H$ 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 $\delta^{2}H$ 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 $^{2}H/^{1}H$ signatures.

87 Other studies have focused on the analysis of modelled and/or empirical leaf water and *n*-
88 alkyl lipid $^{2}H/^{1}H$ compositions to avoid the limitations inherent in the above approach.
89 McInerney et al. (2011) examined the impact of relative humidity on leaf wax $\delta^{2}H$ by
90 analysing *n*-alkanes from grasses grown both in controlled environmental chambers and
91 across a range of climatically different field sites. Modelled leaf water $\delta^{2}H$ values, however,
92 were more positive than would have been expected from empirical *n*-alkane $\delta^{2}H$ data.
93 McInerney et al. (2011) suggested that ^{2}H-enriched leaf waters were not the biosynthetic
94 precursor for leaf wax synthesis, as the best correlation between source water and lipid $\delta^{2}H$
95 values was obtained though using 100% xylem water. The potential for biochemical
96 mechanisms to explain differences in fractionation between C_3 and C_4 plants was mentioned,
97 but the design of the study did not allow for assessment of its relative importance. Sachse et
98 al. (2010) also focused on monocot species, analysing field-grown barley (*Hordeum vulgare*)
99 across one growing season. This study found a correlation between midday leaf water and *n*-
100 C_{31} alkane $\delta^{2}H$ values. However, their model, which assumed a 1:1 relationship between leaf
101 water (source) and leaf wax (product), overestimated ^{2}H-enrichment of the *n*-C_{31} alkane. The
102 authors proposed that this discrepancy could be due to a ^{2}H-depleted pool of water used
103 during biosynthesis, which may have originated from spatial inhomogeneity in ^{2}H-
104 enrichment along the length of a leaf. This study did not address the question of whether
105 biochemical mechanisms might explain the lack of a 1:1 relationship between source water
106 and *n*-alkane $^{2}H/^{1}H$.
107
108 The potential for biochemical processes to influence leaf wax $^{2}H/^{1}H$ has been considered
109 previously in limited circumstances. Kahmen et al. (2013) investigated whether evaporative
110 ^{2}H-enrichment in leaf water was recorded in the leaf waxes of five angiosperm species grown
111 under controlled growth chamber conditions. The results of this study suggested that the

112 influence of evaporative ^{2}H-enrichment was species-specific; with 18 to 68% of the leaf
113 water ^{2}H-enrichment reflected in *n*-alkanes. However, interspecies variation of up to 65‰
114 was observed in $^{2}H/^{1}H$ fractionation between xylem water and *n*-alkanes. This range in
115 fractionation could not be attributed to differences in measured leaf water evaporative ^{2}H-
116 enrichment among the studied species. The authors, therefore, theorised that species-specific
117 variation in NADPH sources used for lipid biosynthesis could have been the reason for this
118 variation. Sessions (2006) studied seasonal shifts in the C_4 saltmarsh grass *Spartina*
119 *alterniflora,* growing in seawater, which were assumed to have the same isotopic composition
120 throughout the sampling period. The relative ^{2}H-depletion in lipid $^{2}H/^{1}H$ observed during the
121 summer months – contrary to the anticipated ^{2}H-enrichment in summer – was interpreted as a
122 change in the organic substrate used for lipid biosynthesis, i.e. current photosynthate in
123 summer, versus stored carbohydrates during the winter. Feakins and Sessions (2010)
124 considered whether changes in the source of biosynthates influenced species-specific
125 variation in $^{2}H/^{1}H$ among CAM plants. Hydrogen isotope fractionation between source water
126 and *n*-alkanes differed by 92‰ among species. However, the authors had not measured
127 xylem or leaf water $\delta^{2}H$ as part of this study, but theorised that these differences may have
128 arisen from metabolic moderation of fractionation between leaf water and leaf wax by using a
129 percentage of NADPH generated from heterotrophic pathways for lipid biosynthesis.
130
131 Remarkably, the inadequacy of relying solely on physical mechanisms to explain leaf water
132 empirical $\delta^{2}H$ data was shown by Shu et al. (2008), who modelled leaf water oxygen and
133 hydrogen isotope compositions along the length of a pine needle. Even though their model
134 could describe along-leaf variation in empirical $\delta^{18}O$ data, it could not do it for $\delta^{2}H$ data. The
135 authors proposed that this discrepancy was due to the fact that "certain unknown biological
136 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 δ^2H values required a new approach that integrated $^2H/^1H$ 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 $^2H/^1H$ 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 is 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 $^2H/^1H$ 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 δ^2H 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 δ^2H 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

187 Stiffkey marsh is typical of an open coast back-barrier saltmarsh (Moeller et al., 1996; Allen,
188 2000) (Fig. 1). The site can be divided into ecologically distinct zones. The low marsh (LM)
189 and upper marsh (UM), defined by Jeffries (1977), are separated by a well-drained gravel and
190 sand ridge (R, Fig. 1) formed by onshore emplacement of offshore barrier sediments (Boomer
191 and Woodcock 1999). Seawater inundation onto the upper marsh is by tidal flow through a
192 dendritic channel network across the marsh and also by spring tidal inundation. Neap tides
193 range from 2 to 3 m, although they can be as low as 0.2 m (Pye, 1992; Callaway et al., 1996).
194 Spring tides can be in excess of 5 m and storm surges from the North Sea can occur
195 (Callaway et al., 1998; Andrews et al., 2000). There are no rivers or streams draining onto the
196 marsh, therefore rainwater accounts for all near-surface fresh water inputs to the site.
197
198 **2.2 Surface vegetation**
199
200 Stiffkey vegetation cover can be zoned according to topography and degree of tidal
201 inundation (Jeffries, 1977; Jeffries and Perkins, 1977; Davy et al., 2011). Plant types include
202 grasses (*Spartina anglica, Elytrigia atherica, Phragmites australis, Puccinella maritima*),
203 succulents (*Suaeda vera, Salicornia europaea*) and dicots (*Limionium vulgare, Atriplex*
204 *portulacoides*). The low marsh at Stiffkey, which receives regular tidal inundation, is
205 colonised by C_4 grass *Spartina anglica,* C_3 annuals *Salicornia europaea* and *Limonium*
206 *vulgare,* and occasionally the C_3 shrub *Atriplex portulacoides*. The gravel ridge supports a
207 range of C_3 grasses such as *Elytrigia atherica,* with stands of the reed *Phragmites australis*
208 found on the seaward side. *Suaeda vera* and *Atriplex portulacoides* also grow in ≤1 m high
209 bushes on the ridge. *Limonium vulgare, Atriplex portulacoides* and *Suaeda vera* are
210 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 europea* (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 $^{2}H/^{1}H$ (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 $^{2}H/^{1}H$ compositions. These three species were chosen because of their close proximity to each other and because they showed the maximum range in *n*-alkane $\delta^{2}H$ 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 europea*: 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 ºC 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 ºC 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 $^{2}H/^{1}H$ 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 $\delta^{2}H$ 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 affects associated with the use of liquid autosampler. The $\delta^{2}H$ 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 $^{2}H/^{1}H$ 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 $\delta^{2}H$ 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.
313
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 ºC to 150 ºC at 20 ºC min^{-1}, and then at 8 ºC
328 min^{-1} to 320 ºC (10 min). *n*-Alkanes were identified by comparison of their elution times with
329 *n*-C_{16} to *n*-C_{30} 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).
332
333 **3.4 *n*-Alkane hydrogen isotope analysis**
334

335 The $^{2}H/^{1}H$ composition of *n*-alkanes was determined using a Delta V Advantage
336 ThermoFisher isotope-ratio mass spectrometer interfaced with GC-Isolink Trace GC
337 Combustion and High temperature conversion (HTC) system operating at 1420 ºC. The initial
338 GC oven temperature was set at 50 ºC, which was then raised at a rate of 30 ºC min^{-1} to 220
339 ºC. A second temperature ramp to a final temperature of 320 ºC at a rate of 6 ºC min^{-1}
340 followed. The final temperature was held for 5 min. The $\delta^{2}H$ values are based on duplicate
341 analyses of well-resolved peaks and reported on the VSMOW scale, based on in-house
342 reference gases (H_2, >99.995% purity, BOC) adjusted at the beginning and at the end of each
343 sequence using a standard mixture of the *n*-C_{16} to *n*-C_{30} alkane standard. Root mean square
344 (RMS) errors for $^{2}H/^{1}H$ measurements of this standard were 4.0‰ (n = 780). During all
345 sample and standard measurements, six reference gas pulses were passed through the mass
346 spectrometer. Reproducibility of H_2 reference gas $\delta^{2}H$ values after H_3^+ correction was ±6‰.
347 Typical absolute differences in *n*-C_{29} measurements between analytical replicates of the same
348 sample did not exceed 6‰, while absolute differences in mean values among sample
349 replicates of the same species (an indicator of intraspecies variability) was on average 4‰,
350 with a maximum of 10-14‰ for *Atriplex portulacoides* (August 2012), *Phragmites australis*
351 (September 2012) and *Suaeda vera* (September 2012) (Supplementary Information Table 4).
352
353 **4. RESULTS**
354
355 **4.1 Soil water $^{2}H/^{1}H$ composition**
356
357 Soil water from the sandflat was most ^{2}H-depleted in March (-27‰) and most ^{2}H-enriched in
358 May (+2‰) (SI Table 4). Between May and September 2012, soil water from the sandflat
359 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‰ ^{2}H-depleted compared with samples collected between 12:00-14:00 (Supplementary Information Table 5).

4.2 Xylem water $^{2}H/^{1}H$ 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 $\delta^{2}H$ 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 $^{2}H/^{1}H$ 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 $^{2}H/^{1}H$ composition

384 Leaf waters extracted from all species collected at Stiffkey in June 2011 varied by no more
385 than 29‰. For those species sampled from multiple locations, upper marsh leaf water
386 samples were generally more ^{2}H-enriched than those sampled from other locations, but the
387 range of variation was low compared to gross interspecies differences: $\delta^{2}H_{LW}$ from *Atriplex*
388 *Portulacoides* varied by 13‰ across the marsh, with the most ^{2}H-depleted leaf water found at
389 the ridge site and the most ^{2}H-enriched in the upper marsh; $\delta^{2}H_{LW}$ from *Triglochin maritima*
390 varied by 10‰ between the lower and upper marsh. Small shifts of 6‰ were observed in the
391 evergreen succulent *Suaeda vera*, and the perennial herb *Limonium vulgare,* with the most
392 ^{2}H-enriched value occurring in the upper marsh for *Suaeda* and the lower marsh for
393 *Limonium* (Fig. 4; Supplementary Information Table 3).
394
395 Leaf water samples collected during 2012 showed a total range among all species sampled of
396 46‰ between the most ^{2}H-depleted values (-26‰, *Limonium vulgare,* March) and the most
397 ^{2}H-enriched (+20‰, *Salicornia europaea,* September). Species-specific variation in leaf
398 water $\delta^{2}H$ was most limited in March (6‰) and greatest in August (29‰). Leaf waters from
399 all species were generally most ^{2}H-depleted in March, and ^{2}H-enriched in September.
400 *Elytrigia atherica* and *Phragmites australis* were generally the most ^{2}H-depleted in terms of
401 leaf water $\delta^{2}H$, whilst *Spartina anglica, Limonium vulgare* and *Salicornia europaea* were
402 typically among the most ^{2}H-enriched. The exception to this overall pattern among species
403 occured in March, when all species were characterized by $\delta^{2}H$ values between -26‰ and -
404 20‰ (Fig. 5; Supplementary Information Table 4). These extremely negative leaf water $\delta^{2}H$
405 profiles were significantly different (Minitab v.16, 2013, student's t-test, $P>0.05$, n = 10
406 individuals per sampling interval comparing those species growing from March to September
407 2012) to those observed for the same species in all other sampling intervals during 2012.
408

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 C_3 grass *Elytrigia atherica* showed the greatest shift in the δ^2H 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 $^2H/^1H$ 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 C_4 grass *Spartina anglica,* and the C_3 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 C_3 monocot *Elytrigia atherica*.

4.4 *n*-Alkane $^2H/^1H$ composition

Analysis of molecular distributions of *n*-alkanes from the sampled species (Supplementary Information Table 1 and 2) showed that *n*-C_{27} and *n*-C_{29} alkanes were the most abundant across all species. Because *n*-C_{27} and *n*-C_{29} alkane δ^2H values were strongly correlated across the growing season (Fig. 2 in the Supplementary Information), we focused only on *n*-C_{29} δ^2H values in all subsequent data analysis. The mean *n*-C_{29} δ^2H values from June 2011 showed a

434 total interspecies variation of 98‰, with the C_3 grass *Elytrigia atherica* having the most ^{2}H-
435 depleted *n*-C_{29} value and *Suaeda vera* the most ^{2}H-enriched. Species collected from multiple
436 sampling sites showed very limited micro-habitat dependent variation ranging from 1‰ (i.e.
437 below the observed maximum intraspecies variability of 6‰ in *n*-C_{29} ^{2}H/^{1}H) (*Suaeda vera*) to
438 9‰ (*Atriplex portulacoides*). The greatest interspecies range in $\delta^2H_{n\text{-}C29}$ was observed at the
439 ridge site (93‰), while the lowest occurred in the upper marsh (24‰). *n*-C_{29} from C_3 grasses
440 was on average 45‰ more ^{2}H-depleted than that from the C_4 *Spartina anglica*. Overall, we
441 observed the following pattern for *n*-C_{29} alkane δ^2H values: succulents > perennial herbs >
442 evergreen shrubs > C_4 grass > C_3 monocots (Fig. 4; Supplementary Information Table 3).
443
444 The mean δ^2H values of *n*-C_{29} alkane from the 2012 growing season were remarkably
445 consistent for each individual species across all the sampling intervals (Fig. 5; Supplementary
446 Information Table 4). Seasonal variation from March – September was the highest in the
447 evergreen succulent *Suaeda vera* (44‰), and the lowest in the annual succulent *Salicornia*
448 *europaea* (5‰). For all other species, seasonal variation in their leaf wax ^{2}H/^{1}H composition
449 fell within the range of 10-35‰. Statistical analysis (Minitab v.16, 2013) confirmed that
450 these differences are not significant (Mann-Whitney U test, $P>0.05$, $n=10$ for March 2012;
451 $n=14$ for May, August and September 2012).
452
453 The greatest interspecies variation in *n*-C_{29} occurred in August (120‰), however variability
454 among species exceeded 100‰ for all 2012 study intervals. *Elytrigia atherica* and
455 *Phragmites australis* consistently recorded the most negative δ^2H values. However, unlike in
456 the leaf water – where *Phragmites* was generally more negative than *Elytrigia* – the *n*-C_{29}
457 alkane δ^2H values of *Elytrigia* were between 23 and 54‰ more negative than those of
458 *Phragmites* across the entire growing season. In addition, the most ^{2}H-enriched *n*-C_{29} values

were observed in *Suaeda vera, Limonium vulgare* and *Salicornia europaea,* with *Spartina anglica* – a species with one of the more positive leaf water δ^2H values – having intermediate *n*-C_{29} alkane δ^2H values across all sampling intervals (Fig. 5). Cross-plotting the *n*-C_{29} alkane δ^2H 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 $^2H/^1H$ 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 $^2H/^1H$ fractionation between soil, xylem and leaf water and *n*-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): $\Delta^2H = \delta^2H_{soil\ water} - \delta^2H_{xylem\ water.}$

Δ^2H 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 Δ^2H 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 $^{2}H/^{1}H$ fractionation between mean *n*-C_{29} $\delta^{2}H$ values and soil water ($\varepsilon_{wax/sw}$), xylem water ($\varepsilon_{wax/xw}$), and leaf water ($\varepsilon_{wax/lw}$) using the following equation:

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

where $\delta^{2}H_{water}$ 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*-C_{29} and leaf water exceeded 100‰ (Fig. 7a & 7b). Similar differences were identified throughout the 2012 growing season when the total variation in $\varepsilon_{wax/lw}$ exceeded 86‰ for all sampling intervals (Fig. 8). The greatest range in $\varepsilon_{wax/lw}$ 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 $\varepsilon_{wax/lw}$ 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 $\varepsilon_{wax/lw}$ 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 $\varepsilon_{wax/lw}$ reported to date for saltmarsh environments (c.f. Romero and Feakins, 2011). $\varepsilon_{wax/sw}$ values

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

The C_4 grass, *Spartina anglica*, has $\varepsilon_{wax/lw}$ values that are higher (by up to 74 ‰) than those observed for the C_3 grass *Elytrigia atherica*. When the *Spartina* data are compared with other C_3 species collected in March and May 2012, $\varepsilon_{wax/lw}$ 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 $\varepsilon_{wax/lw}$ among these species increases: in August, where the maximum variation is observed, the $^2H/^1H$ fractionation between leaf water and leaf wax *n*-C_{29} in *Spartina* is between 25 and 53‰ lower than these other C_3 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 δ^2H 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 δ^2H. 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

528 composition of xylem water, reflecting root uptake of soil water and transport to the leaf, and;
529 (iv) interspecies differences in the isotopic composition of leaf water among plant life forms
530 due to differences in leaf structure, affecting the transpiration of water within the leaf. Each
531 of these scenarios will be considered below, to assess whether they can account for the
532 variation observed in $\delta^2H_{n\text{-}C29}$ among the Stiffkey plants.
533
534 **5.1 The significance of spatial differences in soil water**
535
536 Salt marshes are of great significance in lowland coastal regions (Allen, 2000) and represent
537 important depositional environments because they are divisible into discrete micro-
538 environmental zones based on topography and tidal inundation (Vince and Snow, 1984). This
539 characteristic makes salt marshes ideal for studying plant/environment interactions (Vince
540 and Snow, 1984; Romero and Feakins, 2011). Soils and sediments at Stiffkey receive water
541 inputs from two sources: Sea water, which inundates the lower marsh and low-lying areas of
542 the upper marsh daily, and meteoric precipitation, which is especially important on the ridge
543 where no tidal inundation occurs.
544
545 Previous studies (Romero and Feakins, 2011) show that environmental water varies in
546 isotopic composition across salt marsh sites. Our data from 2012 demonstrates this
547 (Supplementary Information Table 4), showing that the LM and UM (both sites that regularly
548 receive inputs of saline water) have relatively similar isotopic compositions of source water (-
549 2‰ to +2‰ between May and September 2012). Soil water from the ridge at Stiffkey is up to
550 35‰ more ^{2}H-depleted than the other two sampling sites.
551

552 Despite these spatial changes in the isotopic composition of environmental water, large
553 variations in the $\varepsilon_{wax/lw}$ 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*-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 $^{2}H/^{1}H$ 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 $\delta^{2}H$ 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 $\delta^{2}H_{lw}$ values observed across
567 the marsh as a whole (29‰). Differences in mean $\delta^{2}H_{n\text{-}C29}$ 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 $\delta^{2}H_{n\text{-}C29}$ among all sampled plants. In addition, $\varepsilon_{wax/lw}$ 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 $\varepsilon_{wax/lw}$ 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 $^{2}H/^{1}H$ 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 $\delta^{2}H$ signal. There is conflict in previous research over whether the *n*-alkane $^{2}H/^{1}H$ 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 $\delta^{2}H$ 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 $^{2}H/^{1}H$ 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 $\delta^{2}H$ values remained relatively consistent in the mature leaf. This was interpreted to reflect the fixing of the *n*-alkane $\delta^{2}H$ signal during the bud break period, where new waxes are produced from water and stored sugars, suggesting that the *n*-alkane $^{2}H/^{1}H$ 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*-C_{27} – *n*-C_{31} *n*-alkanes are replaced over a timescale of 71-128 days.

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

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

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

626 year (see for example Sessions, 2006). At Site 3 seawater ingress is through a dendritic
627 network of tidal channels (Figure 1), and the proliferation of Triglochin maritima and
628 Salicornia europaea, species known to require saline water, attest to the importance of sea-
629 water inputs to the upper marsh (Allison, 1992; Davy and Bishop, 1991). However, early in
630 the growing season, March soil water δ^2H from the lower marsh shows a considerably more
631 ^2H -depleted value than for other sampling intervals. Examination of local weather station
632 monitoring data (MIDAS, UK Meteorological Office) shows that on the day of sampling
633 rainfall occurred at the site before sampling and after the last high tide. The estimated value
634 for δ^2H of precipitation in North Norfolk in March is c. -62‰ (OIPC), and assuming a
635 seawater δ^2H value of 0‰, we calculate that rainfall contributed ~40% of the ^2H /^1H soil
636 water signal in this sample. It is likely, however, that with the next high tide, the importance
637 of this meteoric water input would be negated. The δ^2H data from May and September 2012
638 support this, as they have a 'near-seawater' isotopic signature, ranging from -2 to +2‰ (SI
639 Table 4). Therefore, regardless of the season during which LM and UM plant species
640 synthesised leaf waxes, temporal isotopic shifts in soil water cannot explain interspecies
641 variation in the *n*-C_{29} alkane δ^2H values observed in these two locations.
642
643 In contrast, the ridge is only rarely inundated by tides and is dominated by meteoric
644 precipitation, which explains why our most ^2H-depleted soil water is found at this site (SI
645 Table 4). Examination of mean monthly interpolated δ^2H values of meteoric water at the
646 Stiffkey site (OIPC; Supplementary Information Table 6) for our sampling periods show,
647 however, modelled precipitation $^2H/^1H$ ranges from -62‰ (March) to -48‰ (September).
648 Soil waters from the ridge are consistently more ^2H-enriched than these meteoric
649 precipitation δ^2H profiles, which we attribute to two likely causes. Firstly, as daytime
650 temperatures rise during the growing season, soil evaporation will increase, particularly from

651 the near-surface depths sampled, resulting in increasing ^{2}H-enrichment in the remaining pore
652 water. Secondly, as the water table at the site is relatively high, an upwards movement of
653 water through soil capillaries ("capillary rise", Plaster, 2009), particularly during warmer
654 summer months, may carry ^{2}H-enriched seawater towards the soil surface (Plaster, 2009).
655 When we consider these temporal shifts in environmental water $^{2}H/^{1}H$ composition in the
656 context of the interspecies variability in leaf wax *n*-alkane hydrogen isotope compositions
657 observed at this particular sampling site, it is clear that temporal variation in the isotopic
658 composition of soil water and precipitation cannot explain the $\delta^{2}H_{n\text{-}C29}$ range among the ridge
659 species. In our study, soil water $\delta^{2}H$ varied by 31‰ at the ridge across the 2012 growth
660 season, while the average interspecies range in $\delta^{2}H_{n\text{-}C29}$ consistently exceeded 100‰.
661
662 In addition to consideration of seasonal shifts in the isotopic composition of environmental
663 water, soil samples collected from the ridge between 7:30 and 8:00 on the 7th of September
664 2012 allowed us to investigate diurnal changes in soil water $\delta^{2}H$. Sachse et al. (2010)
665 suggested that one reason a direct 1:1 relationship was not observed between the $\delta^{2}H$ of
666 midday leaf water and $\delta^{2}H_{n\text{-}C29}$ in barley was that plants were synthesising these compounds
667 from water that had not been subjected to diurnal ^{2}H-enrichment. In our study, the hydrogen
668 isotope signature of soil water from the ridge between 7:30 and 8:00 was 16‰ lower
669 compared with soil samples collected between 12.00 and 14.00 (SI Table 5), while leaf waxes
670 from species sampled at the ridge in September varied by ~90‰. Therefore, diurnal variation
671 in environmental water also cannot explain the range in interspecies $\delta^{2}H_{n\text{-}C29}$ observed in the
672 coastal plants at Stiffkey.
673
674 **5.3 The significance of soil water uptake by halophytes and non-halophytes**
675

Sachse et al. (2010) considered the possibility of a ^{2}H-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 $^{2}H/^{1}H$ fractionation of water molecules, with xylem waters becoming ^{2}H-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 ($\Delta^{2}H$) can explain the variation in $\delta^{2}H_{n\text{-}C29}$ in our data set. $\Delta^{2}H$ 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 $\delta^{2}H_{n\text{-}C29}$ values. Equally, *Atriplex* and *Suaeda* growing on the ridge have the same $\Delta^{2}H$ values (28‰), but their $\delta^{2}H_{n\text{-}C29}$ 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 ^{2}H-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 $\delta^{2}H$ *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 ^{2}H-enrichment to *n*-alkane data have attempted to explain observed variation in *n*-alkane $^{2}H/^{1}H$ 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

726 display very different life forms ranging from succulents, grasses and shrubs. However, leaf
727 waters extracted from morphologically distinct species at the same site in June 2011 (Fig. 4)
728 show very little variation in their δ^2H values. For example, the ridge contains a range of plant
729 species that differ significantly with respect to their leaf morphology. The reed *Phragmites*
730 *australis* has large, elongated leaves up to 30 cm long and 2 cm wide, while the leaf succulent
731 *Suaeda vera* has leaves that are only 3 mm in length and approximately 1.5 mm in diameter.
732 However, the δ^2H_{lw} values range from +5‰ to +21‰ whilst $\delta^2H_{n\text{-}C29}$ values differ by over
733 65‰ between these species. Similar patterns can be found in the seasonal data from 2012,
734 where statistical analysis (Mann-Whitney U test, $P>0.05$, $n= 8$ for comparison of species
735 growing from March to September 2012; $n= 6$ for comparison of species growing from May
736 to September 2012) confirms that interspecies variation in leaf water hydrogen isotope
737 composition is generally not significant. Even if we compare species with extreme variation
738 in leaf morphology such as *Phragmites australis* and *Suaeda vera* –where a statistically
739 significant difference in leaf water does exist – leaf water $^2H/^1H$ between these two plants
740 only ranges from 6 to 12‰ between May and September 2012. Leaf wax $n\text{-}C_{29}$ $^2H/^1H$ values,
741 however, differ consistently by over 50‰ during the same period (Fig. 5).
742
743 When all the species sampled at Stiffkey are considered, variability in leaf water δ^2H
744 composition is three times lower than that observed in $\delta^2H_{n\text{-}C29}$ in June 2011, and consistently
745 4-5 times lower throughout the seasonal time series from 2012. $^2H/^1H$ composition of *n*-
746 alkanes ($\delta^2H_{n\text{-}C29}$) varies across all seasonal sampling periods at Stiffkey by over 100‰, with
747 the greatest variability observed in August (120‰). In contrast, leaf waters across the same
748 period (δ^2H_{lw}) show a total variation of only 29‰ (Supplementary Information Table 4). This
749 contrast between a large variability of *n*-alkane δ^2H and a small range of leaf water δ^2H
750 values is particularly striking at the beginning and mid stages of the growth season. In March

751 2012, the mean values of *n*-C_{29} alkane show 103‰ variation among sampled species, with
752 only 6‰ shifts in leaf water, whilst in August 2012 the *n*-C_{29} range exceeds 120‰ and leaf
753 waters vary by only 29‰. *Phragmites australis* generally has the most negative leaf water
754 $^{2}H/^{1}H$ profile, whilst *Limonium vulgare*, *Spartina anglica* and *Salicornia* have leaf waters
755 that are all generally ^{2}H-enriched compared with other species. Statistical analysis (student's
756 t-test, $P>0.05$, n = 10 individuals per sampling interval comparing those species growing
757 from March to September 2012) of seasonal shifts in leaf water $^{2}H/^{1}H$ among each species
758 shows that March 2012 is significantly different from all other months. The range in leaf
759 water $\delta^{2}H$ in March 2012 is quite limited compared with all other sampling periods. Even if
760 the *n*-alkane $^{2}H/^{1}H$ profiles of our sampled species are in fact fixed at the time of leaf
761 expansion, e.g. as suggested by Tipple et al. (2013), the range in $\delta^{2}H_{n\text{-}C29}$ alkanes observed in
762 March 2012 (103‰) have therefore to be attributed to something other than leaf water
763 isotopic composition.
764
765 In addition, our data also show that ^{2}H-depletion and ^{2}H-enrichment in leaf water and *n*-C_{29}
766 alkane values do not co-vary, i.e. any similarity in leaf water $^{2}H/^{1}H$ composition does not
767 necessarily lead to a similarity in *n*-alkane $\delta^{2}H$ values. Figure 2 presents data from the
768 September 2012 sampling period, and shows that for species with very similar leaf water
769 $^{2}H/^{1}H$ compositions, *n*-alkane values can vary considerably. For example, whilst *Limonium*
770 *vulgare* and *Salicornia* have the most ^{2}H-enriched leaf water and *n*-alkane values, *Atriplex*
771 *portulacoides*, *Suaeda vera* and *Elytrigia atherica* have leaf water values within 8‰ of each
772 other whereas their *n*-alkane values vary by up to 89‰. In addition, the difference between
773 $\delta^{2}H_{lw}$ of *Limonium* and *Elytrigia* is 19‰, while the range in *n*-C_{29} between these species $\delta^{2}H$
774 reaches 105‰.
775

776 Similar discrepancies between the magnitude of differences in the hydrogen isotope
777 composition of leaf waters and the hydrogen isotope composition of the *n*-C_{29} alkane are
778 found throughout all the sampling periods. For example, data collected in June 2011 (Fig. 4;
779 Supplementary Information Table 2) *Triglochin maritima* from the low marsh has the most
780 ^{2}H-depleted leaf water value (+22‰) of plants found in this sub-environment, but this does
781 not result in *Triglochin maritima* having the most ^{2}H-depleted *n*-C_{29} alkane value. Similarly,
782 the C_4 grass *Spartina anglica* has the most ^{2}H-depleted *n*-C_{29} alkane (-156‰) value in the low
783 marsh, but one of the more ^{2}H-enriched leaf waters (+27‰). This lack of correlation between
784 leaf water and leaf wax $\delta^{2}H$ at the plant species level is also apparent in the June 2011 dataset
785 when species having very similar leaf water values – *Limonium vulgare* and *Salicornia*
786 *europaea* differ by only 1‰ in the low marsh – synthesized *n*-C_{29} alkanes that differ by as
787 much as 20‰ (Fig. 4).
788
789 At the ridge, where the greatest range in $\varepsilon_{wax/lw}$ values is observed in June 2011, this lack of
790 correlation between leaf water and *n*-C_{29} alkane $^{2}H/^{1}H$ composition is also present (Fig. 4).
791 Here, it is the C_3 reed, *Phragmites australis* that has the most ^{2}H-depleted leaf water (+5‰),
792 but the *n*-C_{29} *n*-alkane $\delta^{2}H$ value for this species does not follow this trend (Fig. 4). The most
793 ^{2}H-depleted *n*-C_{29} alkane value on the ridge is in fact found in another C_3 grass, *Elytrigia*
794 *atherica*, which has a leaf water $\delta^{2}H$ value of +15‰. As observed in the low marsh, similar
795 leaf water δD values do not result in similar *n*-C_{29} alkane $\delta^{2}H$ values: *Atriplex portulacoides,*
796 and *Suaeda vera* and *Elytrigia atherica* all record leaf water $^{2}H/^{1}H$ values ranging from +15
797 to +21‰, but differ by 93‰ in terms of their *n*-C_{29} alkane $\delta^{2}H$ values. Even in the upper
798 marsh, where the $\delta^{2}H$ values display the smallest overall range among plant species,
799 *Triglochin maritima* and *Atriplex portulacoides* record the highest leaf water $\delta^{2}H$ values but
800 in contrast have lowest *n*-C_{29} alkane $\delta^{2}H$ values (Fig 4). Statistical analysis of interspecies

variation in *n*-C_{29} hydrogen isotope composition supports our finding that leaf water $^2H/^1H$ is of limited relative importance in controlling leaf wax δ^2H values. Variation in midday leaf water δ^2H among the sampled species was not found to be statistically significant, while in contrast interspecies variation in *n*-C_{29} δ^2H was, suggesting some other mechanism was responsible for the >100‰ range in *n*-C_{29} 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*-C_{29} alkane δ^2H 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*-C_{29} alkane δ^2H 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 $^2H/^1H$ *n*-C_{29} value – made use of early morning xylem water (-47‰) for lipid synthesis, while *Suaeda vera* (the species with the highest $^2H/^1H$ *n*-C_{29} 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 $\delta^2H_{n\text{-}C29}$ between them.

5.5 Comparison of $^2H/^1H$ fractionation among C_3 and C_4 plants at Stiffkey with previously published research

Earlier work has suggested that C_3 vs. C_4 plants have relatively invariant fractionation factors between *n*-alkanes and leaf/source water. Examples include the generalised apparent

826 fractionation factors between leaf water and *n*-alkyl lipids calculated for C_3 (-117±27‰) and
827 C_4 (-132±12‰) plants (Chikaraishi and Naraoka, 2003; Chikaraishi et al., 2004), which
828 continue to be applied to modern vegetation studies (Tipple et al., 2013) and palaeoclimate
829 reconstructions (van Soelen et al., 2013; Lieder et al., 2013). Our data suggest these predicted
830 values may not reflect the true extent of plant lipid $^2H/^1H$ diversity - if, for example,
831 fractionation is calculated between leaf water and the *n*-C_{29} alkane for September 2012, only
832 half of the C_3 plants sampled have $\varepsilon_{wax/lw}$ values that fall within the range predicted by
833 Chikaraishi and Naraoka (2003; 2004; Supplementary Information Table 4). The remaining
834 C_3 species, which include *Elytrigia atherica, Phragmites australis* and *Atriplex*
835 *portulacoides*, have $\varepsilon_{wax/water}$ values that are 26-83‰ lower than the predicted values. This
836 lack of agreement with estimated values is found throughout our dataset – in June 2011, only
837 two C_3 species conform to the predicted values (Fig. 7b), while between March and August
838 2012, only *Limonium vulgare*, *Suaeda vera* and *Salicornia europaea* have $\varepsilon_{wax/lw}$ values that
839 regularly fall within the predicted -90 to -144‰ range for C_3 species (Chikaraishi and
840 Naraoka, 2003; 2004). With regards to the C_4-plant group, our calculated $\varepsilon_{wax/lw}$ values for
841 the C_4 grass *Spartina anglica* for both June 2011 (-178‰) and the 2012 growth season (-115
842 to -176‰ between March and September) exceed the range of -120 to -144‰ for C_4 species
843 published by Chikaraishi and Naraoka (2003, 2004; Fig. 7b; Fig. 8).
844
845 A consistent difference in apparent fractionation among C_3 and C_4 species has also been
846 identified in some studies. For example, Chikaraishi and Naraoka (2003) presented data
847 suggesting that C_4 species had higher apparent fractionation factors compared with C_3
848 angiosperms and gymnosperms. However, plant functional types were not distinguished in

849 this study, and large standard deviations for the mean $\varepsilon_{wax/w}$ values (C_3 = -116±25‰, C_4 = -
850 133±12‰) give rise to a degree of overlap in the range of these values. Bi et al. (2005)
851 published data suggesting that in fact C_4 species are typified by *n*-alkane $^2H/^1H$ compositions
852 of -150.4±42.6‰, while that *n*-alkane δ^2H signatures in C_3 species average -175.7±29.5‰.
853 Smith and Freeman (2006) limited their study to C_3 and C_4 grasses, and found that ε values
854 were ~20‰ more negative in C_3 grasses relative to C_4 grasses, resulting in more negative n-
855 alkane $^2H/^1H$ compositions in C_3 grasses. Their result for C_3 and C_4 monocots cannot be
856 explained by gross anatomical differences in leaves and, therefore, it has been hypothesised
857 that differences in the interveinal distance among C_3 and C_4 grasses – alongside difference in
858 the extent of the backflow of enriched water from around the stomata – are responsible for
859 the variation (Smith and Freeman, 2006; Tierney et al., 2010).

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

873 consideration also demonstrates that it may not always be accurate to assume that one plant
874 functional type dictates the magnitude of interspecies variation in *n*-alkane $^{2}H/^{1}H$ at any
875 given location.
876
877 **6. CONCLUSION**
878
879 We have carried out a systematic study of the relationship between the hydrogen isotope
880 composition of soil, xylem and leaf water and the $^{2}H/^{1}H$ of the *n*-C_{29} alkane within a range of
881 halophytic and non-halophytic C_3 and C_4 plants growing at Stiffkey marsh in Norfolk, UK.
882 Our data display significant interspecies variation in fractionation between leaf water and leaf
883 wax, ranging from -79 to -229‰ across the 2012 growing season. The > 100‰ range of our
884 $\delta^{2}H_{n\text{-}C29}$ data, and the 150‰ range in $\varepsilon_{wax/lw}$values, extend beyond the typical values for C_3
885 and C_4 plants put forward in previous studies, We thus infer that reconstruction of
886 palaeohydrological regimes based on estimates such as these may not capture the full
887 complexity of the hydrogen isotope information recorded by these plant groups. The range in
888 our *n*-alkane $\delta^{2}H$ cannot be explained by reference to spatial or temporal shifts in the
889 hydrogen isotope composition of soil, xylem or leaf water. We therefore conclude that
890 environmental and physical mechanisms controlling leaf water isotopic composition cannot
891 fully account for the interspecies variation in our *n*-alkane hydrogen isotope data. Instead, our
892 data show that biochemical mechanisms may play a more important role in controlling
893 interspecies variation in (i) *n*-alkane $^{2}H/^{1}H$ composition, and (ii) fractionation between source
894 water and *n*-alkane $^{2}H/^{1}H$, than abiotic factors.
895
896 Previous research has already identified that biochemical processes may have an important
897 role to play in determining leaf biomarker $^{2}H/^{1}H$. 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 δ^2H 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 $^2H/^1H$ 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 $^2H/^1H$, 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 $^2H/^1H$ 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 ^{137}Cs 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 δ^{13}C analyses of *n*-alkanes
959 extracted from terrestrial and aquatic plants. *Phytochemistry* **63**, 361-371.

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

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

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

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

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

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

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

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

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

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

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

987 Gao., L., Burnier, A., Huang, Y. (2012) Quantifying instantaneous regeneration rates of plant
988 leaf waxes using stable hydrogen isotope labelling. *Rapid Commun. Mass Spectrom.,*
989 **26**, 115–122

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

994 Helliker, B., Ehleringer, J. (2002). Differential ^{18}O enrichment of leaf cellulose in C_3 versus
995 C_4 grasses. *Funct. Plant Biol.* **29**, 435-442.

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

999 Hill, M, Mountford, J. , Roy, D., Bunce, R. 1999 *ECOFACT 2a Technical Annex* 490 -
1000 *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 physiolgical 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.

Pagani, M., Pedentchouk, N., Huber, M., Sluijs, A., Schouten, S., Brinkhuis, H., Damste, J. S. S., Dickens, G. R. and the IODP Expedition 302 Expedition Scientists (2006) Arctic hydrology during global warming at the Palaeocene/Eocene thermal maximum. *Nature* **442**, 671-675.

Pedentchouk, N., Sumner, W., Tipple, B., Pagani, M. (2008) $\delta^{13}C$ and δD compositions of *n*-alkanes from modern angiosperms and conifers: An experimental set up in central Washington State, USA. *Org. Geochem.* **39**, 1066-1071.

Plaster, E. (2009) *Soil Science and Management(5th Ed)*. Delmar Cengage Learning, pp. 142 – 165.

Pye, K. (1992) Saltmarshes on the barrier coastline of north Norfolk, eastern England. In: Allen, J. R. L & Pye, K. (Eds.) *Saltmarshes: Morphodynamics, Conservation and Engineering Significance*, pp. 148-179, Cambridge University Press.

Richardson, C. J. (2000) Freshwater wetlands. In: Barbour, M., Billings, W. (Eds) (2000) *North American Wetlands*. Cambridge University Press, pp. 449 – 501.

Romero, I., C., Feakins, S. J. (2011) Spatial gradients in plant leaf wax D/H across a coastal salt marsh in southern California. *Org. Geochem.* **42**, 618-629.

Sachse, D., Gleixner, G., Wilkes, H., Kahmen, A. (2010) Leaf wax *n*-alkane δD values of field-grown barley reflect leaf water δD values at the time of leaf formation. *Geochim. Cosmochim. Acta* **74**, 6741-6750.

Sachse, D., Radke, J., Gleixner, G. (2004) Hydrogen isotope ratios of recent lacustrine sedimentary *n*-alkanes record modern climate variability. *Geochim. Cosmochim. Acta*, **68**, 4877-4889.

Sachse, D., Radke, J., Gleixner, G. (2006) δD values of individual *n*-alkanes from terrestrial plants along a climatic gradient-Implications for the sedimentary biomarker record. *Org. Geochem.* **37**, 469-483.

Sachse, D., Billault, I., Bowen, G., Chikaraishi, Y., Dawson, T., Feakins, S., Freeman, K., Magill, C., McInerney, F., van der Meer, M., Polissar, P., Robins, R., Sachs, J., Schmidt, H., Sessions, A., White, J., West, J., Kahmen, A. (2012) Molecular paleohydrology: interpreting the hydrogen-isotopic composition of lipid biomarkers from photosynthesising organisms. *Annu. Rev. Earth Planet Sci.* **40***,* 221-249.

Schefuß, E., Schouten, S., Schneider, R. R. (2005) Climatic controls on central African hydrology during the past 20,000 years. *Nature* **437**, 1003-1006.

Schirmer, U., Breckle, S. (1982) The role of bladders for salt removal in some Chenopodiaceae (mainly *Atriplex* species). In: Sen, D., Rajpurohit, K. (Eds.) (1982) *Tasks for vegetation science 2: Contributions to the ecology of halophytes,* Dr W Junk Publishers, pp 215-231.

Sessions, A. L. (2006) Seasonal changes in D/H fractionation accompanying lipid biosynthesis in Spartina alterniflora. *Geochim. Cosmochim. Acta* **70**, 2153-2162.

Shepherd, T., Griffiths, D. (2006) The effects of stress on plant cuticular waxes. *New Phytol.,* **171**, 469 – 499.

Shu, Y., Feng, X., Posmentier, E. S., Sonder, L. J., Faiia, A. M., Yakir, D. (2008) Isotopic studies of leaf water. Part 1: A physically based two-dimensional model for pine needles. *Geochim. Cosmochim. Acta* **72**, 5175-5188.

Smith, F., Freeman, K. (2006) Influence of physiology and climate on δD of leaf wax *n*-alkanes from $C_3$and C_3 grasses. **70**, 1172 – 1187.

Thevs, N., Zerbe, S., Gahlert, F., Midjit, M., Succow, M. (2007) Productivity of reed (Phragmites australis Trin. ex Steud.) in continental-arid NW China in relation to soil, groundwater, and land-use. *J. App. Bot. Food Sci.* **81**, 62-68.

Tierney, J., Russell, J., Huang, Y., Sinninghe Damsté, J., Hopmans, E., Cohen, A. (2008) Northern Hemisphere controls on tropical southeast African climate during the past 60,000 years. *Science* **322**, 252-255.

Tierney, J., Russell, J., Huang Y. (2010) A molecular perspective on Late Quaternary climate and vegetation change in the Lake Tanganyika basin, East Africa. *Quat. Sci. Rev.*, **29**, 787 – 800

Tipple, B., Berke, M., Doman, C., Khachaturyan, S., Ehleringer, J. (2013). Leaf-wax *n*-alkanes record the plant-water environment at leaf flush. *P. Natl. Acad. Sci. USA* **110**, 2659-2664.

Tipple, B., Pagani, M. (2013) Environmental control on eastern broadleaf forest species' leaf wax distributions and D/H ratios. *Geochim. Cosmochim. Acta* **111**, 64-77.

UK Meteorological Office. Met Office Integrated Data Archive System (MIDAS) Land and Marine Surface Stations Data (1853-current), NCAS British Atmospheric Data Centre, 2012, 10th March 2013; Available from http://badc.nerc.ac.uk/view/badc.nerc.ac.uk__ATOM__dataent_ukmo-midas

van Soelen, E., Wagner-Cremer, F., Sinninghe-Damsté., J., Reichart, G. (2013) Reconstructing tropical cyclone frequency using hydrogen isotope ratios of sedimentary *n*-alkanes in northern Queensland, Australia. *Palaeogeogr. Palaeocl.,* **376**, 66 – 72.

Vince, S. W., Snow, A. A. (1984) Plant zonation in an Alaskan salt marsh: I. Distribution, abundance and environmental factors. *J. Ecol.* **72,** 651-667.

Waisel, Y. 1972. *Biology of halophytes*, Academic Press, California.

West, A. G., Patrickson, S. J., Ehleringer, J. R. (2006) Water extraction times for plant and soil materials used in stable isotope analysis. *Rapid Commun. Mass. Sp.* **20**, 1317-1321.

Zhang, Z., Zhao, M., Eglington, G., Lu, H., Huang, C. (2006) Leaf wax lipids as paleovegetational and paleoenvironmental proxies for the Chinese Loess Plateau over 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 δ^2H (black diamonds), xylem water δ^2H (grey squares), leaf water δ^2H (white triangles), and *n*-alkane δ^2H (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 δ^2H 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*-C_{29} alkane δ^2H (black circles) and leaf water δ^2H (white circles) values for all plants sampled across the Stiffkey marsh in June 2011 ("C3" and "C4" refer to plant biochemical pathways). Predicted δ^2H 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 $^{2}H/^{1}H$ composition estimated using the Online Isotopes in Precipitation Calculator (Bowen et al., 2005).

Figure 5: Seasonal variation in *n*-C_{29} alkane $\delta^{2}H$ and leaf water $\delta^{2}H$ 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*-C_{29} alkane and 2‰ for leaf water.

Figure 6: Measured leaf water $\delta^{2}H$ 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 ($\varepsilon_{wax/lw}$ ‰) between *n*-C_{29} alkane $\delta^{2}H$ and leaf water $\delta^{2}H$ 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 ($\varepsilon_{wax/lw}$ ‰) between *n*-C_{29} alkane $\delta^{2}H$ and leaf water $\delta^{2}H$ 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.

SI Figure 2: Bivariate plot of ACL and *n*-C_{29} alkane δ^2H (September 2012) showing no correlation between the two parameters. Letters in parenthesis denote plant species: AP = *Atriplex portulacoides,* EA = *Elytrigia atherica,* LV = *Limonium vulgare,* PA = *Phragmites australis,* SE = *Salicornia europaea,* SA = *Spartina anglica,* SV = *Suaeda vera*.

SI Figure 3: Bivariate plot of *n*-C_{27} and *n*-C_{29} alkane δ^2H values for all species sampled across the 2012 growth season showing a strong correlation between the two sets of data.