

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

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

3

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12

## ABSTRACT

13  
14  
15 Interpretation of sedimentary *n*-alkyl lipid  $\delta^2\text{H}$  data is complicated by a limited understanding  
16 of factors controlling interspecies variation in biomarker  $^2\text{H}/^1\text{H}$  composition. To distinguish  
17 between the effects of interrelated environmental, physical and biochemical controls on the  
18 hydrogen isotope composition of *n*-alkyl lipids, we conducted linked  $\delta^2\text{H}$  analyses of soil  
19 water, xylem water, leaf water and *n*-alkanes from a range of  $\text{C}_3$  and  $\text{C}_4$  plants growing at a  
20 UK saltmarsh (i) across multiple sampling sites, (ii) throughout the 2012 growing season, and  
21 (iii) at different times of the day. Soil waters varied isotopically by up to 35‰ depending on  
22 marsh sub-environment, and exhibited site-specific seasonal shifts in  $\delta^2\text{H}$  up to a maximum  
23 of 31‰. Maximum interspecies variation in xylem water was 38‰, while leaf waters differed  
24 seasonally by a maximum of 29‰. Leaf wax *n*-alkane  $^2\text{H}/^1\text{H}$ , however, consistently varied by  
25 over 100‰ throughout the 2012 growth season, resulting in an interspecies range in the  
26  $\epsilon_{\text{wax/leaf water}}$  values of -79 to -227‰. From the discrepancy in the magnitude of these isotopic  
27 differences, we conclude that mechanisms driving variation in the  $^2\text{H}/^1\text{H}$  composition of leaf  
28 water, including (i) spatial changes in soil water  $^2\text{H}/^1\text{H}$ , (ii) temporal changes in soil water  
29  $^2\text{H}/^1\text{H}$ , (iii) differences in xylem water  $^2\text{H}/^1\text{H}$ , and (iv) differences in leaf water evaporative  
30  $^2\text{H}$ -enrichment due to varied plant life forms, cannot explain the range of *n*-alkane  $\delta^2\text{H}$  values  
31 we observed. Results from this study suggests that accurate reconstructions of palaeoclimate  
32 regimes from sedimentary *n*-alkane  $\delta^2\text{H}$  require further research to constrain those biological  
33 mechanisms influencing species-specific differences in  $^2\text{H}/^1\text{H}$  fractionation during lipid  
34 biosynthesis, in particular where plants have developed biochemical adaptations to water-  
35 stressed conditions. Understanding how these mechanisms interact with environmental  
36 conditions will be crucial to ensure accurate interpretation of hydrogen isotope signals from  
37 the geological record.

38

39

## 1. INTRODUCTION

40

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

53

54 Palaeoclimatic reconstructions of source water isotopic composition (Pagani et al., 2006;  
55 Tierney et al., 2008) and moisture availability and aridity (Scheffuß et al., 2005; Leider et al.,  
56 2013) have often implicitly and/or explicitly relied on the assumption that the biosynthetic  
57  $^2\text{H}/^1\text{H}$  fractionation that takes place between the intracellular water and lipids within the plant  
58 is relatively invariant within  $\text{C}_3$  and  $\text{C}_4$  plant groups. The magnitude of variability in the  $\delta^2\text{H}$   
59 values of *n*-alkyl lipids among plant species growing at the same geographical location  
60 suggests, however, that this assumption may not necessarily be valid. Interpretation of  
61 sedimentary *n*-alkyl  $\delta^2\text{H}$  data is further complicated by limited understanding of the reasons  
62 for this large interspecies variability. Sachse et al. (2012) provided a comprehensive review

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

70

71 Previous research has mainly focused on using empirical and modelling studies to investigate  
72 various physical processes that control source and intracellular water. First, there were studies  
73 (e.g. Hou et al, 2007; Pedentchouk et al, 2008) in which a range of plants were considered,  
74 but coupled leaf water and *n*-alkane  $^2\text{H}/^1\text{H}$  measurements were not conducted. Instead, these  
75 studies relied on isotopic measurements of environmental water and leaf wax *n*-alkyl  
76 compounds, and any differences in  $^2\text{H}/^1\text{H}$  fractionation were explained by reference to the  
77 physical processes that controlled the movement of water molecules inside, outside and  
78 within the leaf according to leaf-water models (Farquhar and Lloyd, 1993; Barbour et al.,  
79 2000; Barbour et al., 2004). The implicit assumption of these models (initially developed for  
80 understanding oxygen isotope systematics of plant water) is that they can fully describe  
81 hydrogen isotope systematics of leaf water, and thus also account for the differences in the  
82  $\delta^2\text{H}$  values of leaf wax lipids among different species. The lack of actual measurements of  
83 leaf water isotopic composition, however, prevents such studies from evaluating the relative  
84 importance of physical and biochemical factors that control leaf water and biosynthate  $^2\text{H}/^1\text{H}$   
85 signatures.

86

87 Other studies have focused on the analysis of modelled and/or empirical leaf water and *n*-  
88 alkyl lipid  $^2\text{H}/^1\text{H}$  compositions to avoid the limitations inherent in the above approach.  
89 McInerney et al. (2011) examined the impact of relative humidity on leaf wax  $\delta^2\text{H}$  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  $\delta^2\text{H}$  values, however,  
92 were more positive than would have been expected from empirical *n*-alkane  $\delta^2\text{H}$  data.  
93 McInerney et al. (2011) suggested that  $^2\text{H}$ -enriched leaf waters were not the biosynthetic  
94 precursor for leaf wax synthesis, as the best correlation between source water and lipid  $\delta^2\text{H}$   
95 values was obtained though using 100% xylem water. The potential for biochemical  
96 mechanisms to explain differences in fractionation between  $\text{C}_3$  and  $\text{C}_4$  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  $\text{C}_{31}$  alkane  $\delta^2\text{H}$  values. However, their model, which assumed a 1:1 relationship between leaf  
101 water (source) and leaf wax (product), overestimated  $^2\text{H}$ -enrichment of the *n*- $\text{C}_{31}$  alkane. The  
102 authors proposed that this discrepancy could be due to a  $^2\text{H}$ -depleted pool of water used  
103 during biosynthesis, which may have originated from spatial inhomogeneity in  $^2\text{H}$ -  
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  $^2\text{H}/^1\text{H}$ .

107

108 The potential for biochemical processes to influence leaf wax  $^2\text{H}/^1\text{H}$  has been considered  
109 previously in limited circumstances. Kahmen et al. (2013) investigated whether evaporative  
110  $^2\text{H}$ -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  $^2\text{H}$ -enrichment was species-specific; with 18 to 68% of the leaf  
113 water  $^2\text{H}$ -enrichment reflected in *n*-alkanes. However, interspecies variation of up to 65%  
114 was observed in  $^2\text{H}/^1\text{H}$  fractionation between xylem water and *n*-alkanes. This range in  
115 fractionation could not be attributed to differences in measured leaf water evaporative  $^2\text{H}$ -  
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  $\text{C}_4$  saltmarsh grass *Spartina*  
119 *alterniflora*, growing in seawater, which were assumed to have the same isotopic composition  
120 throughout the sampling period. The relative  $^2\text{H}$ -depletion in lipid  $^2\text{H}/^1\text{H}$  observed during the  
121 summer months – contrary to the anticipated  $^2\text{H}$ -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  $^2\text{H}/^1\text{H}$  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  $\delta^2\text{H}$  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  $\delta^2\text{H}$  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  $\delta^{18}\text{O}$  data, it could not do it for  $\delta^2\text{H}$  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

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

142

143 As a result of all the previous research we can therefore hypothesize that if interspecies  
144 differences in the  $^2\text{H}/^1\text{H}$  composition of leaf wax lipids are driven primarily by differences in  
145 the isotopic composition of leaf water, there are several theoretical scenarios that may  
146 account for the observed variability among plant species growing at the same site. These  
147 include: (i) differences in the isotopic composition of soil water among site sub-  
148 environments; (ii) differences in the isotopic composition of soil water throughout the growth  
149 season; (iii) interspecies differences in xylem water reflecting root uptake of soil water is and  
150 transport to the site of evaporation in the leaf, and (iv) interspecies differences in the isotopic  
151 composition of leaf water among plant life forms due to differences in leaf structure. The  
152 focus of this paper is to test all of these scenarios and to evaluate whether they provide a  
153 comprehensive explanation for differences in the  $^2\text{H}/^1\text{H}$  composition of lipids from a range of  
154  $\text{C}_3$  and  $\text{C}_4$  plant species (grasses, succulents, evergreens and perennial herbs) sampled at  
155 Stiffkey salt marsh, Norfolk, UK across the entire growing season from March to September  
156 in 2012. The broad range of plant life forms was specifically chosen due to (a) their gross  
157 variation in leaf morphology, and (b) their well-studied differences in biochemical  
158 adaptations to their environment, which provided an ideal platform to test the relative  
159 importance of physical and biochemical mechanisms in explaining interspecies variation in  
160 the  $\delta^2\text{H}$  values of leaf wax *n*-alkanes in terrestrial plant species growing in a geographically  
161 restricted natural environment.

162

163 In this study, we focus on a saltmarsh environment at the land/sea divide. These ecosystems  
164 contribute significant amounts of organic material to the marine environment (Mitsch and  
165 Gosselink, 2000). Indeed, globally saltmarshes are known to have higher levels of primary  
166 production than other coastal biomes such as mangroves, and greatly exceed the productivity  
167 of grasslands, cultivated plant communities and forest ecosystems (Mitsch and Gosselink,  
168 2000; Richardson, 2000), with ~50% of organic carbon in ocean sediments being derived  
169 from vegetated sedimentary environments (Duarte et al., 2005). Findings from this study will  
170 therefore have important implications for palaeoclimate reconstructions based on the  $\delta^2\text{H}$   
171 profiles of leaf wax lipids from coastal and marine sediments. In addition, biochemical  
172 adaptations employed by the selected species at Stiffkey to ameliorate water stress are not  
173 unique to saltmarsh settings - other xeromorphic plant species growing in a variety of other  
174 water stressed habitats such as arid regions, are also known to make use of similar  
175 biochemical responses to maintain their osmotic potential (Bohnert and Jensen, 1996), and  
176 thus the conclusions can be translated to such other environments. Understanding the relative  
177 importance of biochemistry in controlling the hydrogen isotope composition of leaf wax  
178 biomarkers in plant biochemical mechanisms is therefore important for helping in the  
179 reconstruction of past climates across a range of different biomes. The data presented here  
180 thus allows us to make far-reaching inferences regarding the interaction between physical and  
181 biochemical mechanisms across a wide variety of plant life forms.

182

183

## 2. STUDY LOCATIONS AND SAMPLING METHODS

184

185

### 2.1 Study location

186

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 (*Limonium vulgare*, *Atriplex*  
204 *portulacoides*). The low marsh at Stiffkey, which receives regular tidal inundation, is  
205 colonised by C<sub>4</sub> grass *Spartina anglica*, C<sub>3</sub> annuals *Salicornia europaea* and *Limonium*  
206 *vulgare*, and occasionally the C<sub>3</sub> shrub *Atriplex portulacoides*. The gravel ridge supports a  
207 range of C<sub>3</sub> 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  $\leq 1$  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*

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

213

214 The distribution of coastal plants at Stiffkey can be explained by considering ‘Ellenberg’  
215 values for salinity tolerance produced as part of the 1999 ‘Ecofact’ project (Hill et al., 1999).

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

233

### 234 **2.3 Sampling strategy**

235

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

248

249 In June 2011 samples were collected for paired leaf wax and leaf water analysis. During  
250 2012, however, sampling also included soil water samples for the entire growing season. In  
251 September 2012, we sampled xylem water as well as leaves from all species between 12:00  
252 and 14:00. In addition we collected soil, leaf and xylem water from *Elytrigia atherica*,  
253 *Suaeda vera*, and *Atriplex portulacoides* at the ridge between 7:30 and 8:00 to allow for  
254 investigation of the potential influence of diurnal shifts in xylem and leaf water on *n*-alkane  
255  $^2\text{H}/^1\text{H}$  compositions. These three species were chosen because of their close proximity to  
256 each other and because they showed the maximum range in *n*-alkane  $\delta^2\text{H}$  values among  
257 species at one sampling site.

258

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

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

274

### 275 3. ANALYTICAL METHODS

276

#### 277 3.1 Leaf, xylem and soil water extraction

278

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

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

290

### 291 **3.2 Water isotopic analysis**

292

293 Hydrogen isotope signatures of extracted waters were measured using a Delta XP  
294 ThermoFisher isotope-ratio mass spectrometer interfaced with a pyrolysis TC/EA equipped  
295 with a liquid autosampler. The  $\delta^2\text{H}$  values reported here are based on ten analytical replicates  
296 of each sample. The first five replicates of each sample were discarded to prevent distortion  
297 by memory affects associated with the use of liquid autosampler. The  $\delta^2\text{H}$  values are  
298 expressed relative to the VSMOW scale based upon analysis of a suite of international and  
299 in-house standards analyzed in the same sequence with the water samples. Additional  
300 standards (GISP, in-house tap water) were treated as unknowns to evaluate instrument  
301 accuracy. Root mean square (RMS) errors for  $^2\text{H}/^1\text{H}$  measurements of international and in-  
302 house standards were 1.0‰ ( $n = 108$ ). During all sample and standard measurements, three  
303 reference gas pulses were passed through the mass spectrometer. Reproducibility of  $\text{H}_2$   
304 reference gas  $\delta^2\text{H}$  values after  $\text{H}_3^+$  correction was typically  $\pm 0.5\%$ . Typical standard error  
305 among analytical replicates of the same sample was 4‰, while comparison of mean values  
306 for leaf and xylem sample duplicates showed that the absolute difference between them was  
307 in all cases also less than 4‰. Soil sample duplicates could not be successfully processed for  
308 all sampling intervals due to difficulties in extracting sufficient amounts of water from them  
309 for reliable stable isotope measurements (Supplementary Information Table 4). However  
310 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 °C to 150 °C at 20 °C min<sup>-1</sup>, and then at 8 °C  
328 min<sup>-1</sup> to 320 °C (10 min). *n*-Alkanes were identified by comparison of their elution times with  
329 *n*-C<sub>16</sub> to *n*-C<sub>30</sub> 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  $^2\text{H}/^1\text{H}$  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 °C. The initial  
338 GC oven temperature was set at 50 °C, which was then raised at a rate of 30 °C min<sup>-1</sup> to 220  
339 °C. A second temperature ramp to a final temperature of 320 °C at a rate of 6 °C min<sup>-1</sup>  
340 followed. The final temperature was held for 5 min. The  $\delta^2\text{H}$  values are based on duplicate  
341 analyses of well-resolved peaks and reported on the VSMOW scale, based on in-house  
342 reference gases ( $\text{H}_2$ , >99.995% purity, BOC) adjusted at the beginning and at the end of each  
343 sequence using a standard mixture of the *n*-C<sub>16</sub> to *n*-C<sub>30</sub> alkane standard. Root mean square  
344 (RMS) errors for  $^2\text{H}/^1\text{H}$  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  $\text{H}_2$  reference gas  $\delta^2\text{H}$  values after  $\text{H}_3^+$  correction was  $\pm 6\%$ .  
347 Typical absolute differences in *n*-C<sub>29</sub> 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

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

356

357 Soil water from the sandflat was most  $^2\text{H}$ -depleted in March (-27‰) and most  $^2\text{H}$ -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

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

367

#### 368 **4.2 Xylem water <sup>2</sup>H/<sup>1</sup>H composition**

369

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

381

#### 382 **4.3 Leaf water <sup>2</sup>H/<sup>1</sup>H composition**

383

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 <sup>2</sup>H-enriched than those sampled from other locations, but the  
387 range of variation was low compared to gross interspecies differences: δ<sup>2</sup>H<sub>LW</sub> from *Atriplex*  
388 *Portulacoides* varied by 13‰ across the marsh, with the most <sup>2</sup>H-depleted leaf water found at  
389 the ridge site and the most <sup>2</sup>H-enriched in the upper marsh; δ<sup>2</sup>H<sub>LW</sub> 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 <sup>2</sup>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 <sup>2</sup>H-depleted values (-26‰, *Limonium vulgare*, March) and the most  
397 <sup>2</sup>H-enriched (+20‰, *Salicornia europaea*, September). Species-specific variation in leaf  
398 water δ<sup>2</sup>H was most limited in March (6‰) and greatest in August (29‰). Leaf waters from  
399 all species were generally most <sup>2</sup>H-depleted in March, and <sup>2</sup>H-enriched in September.  
400 *Elytrigia atherica* and *Phragmites australis* were generally the most <sup>2</sup>H-depleted in terms of  
401 leaf water δ<sup>2</sup>H, whilst *Spartina anglica*, *Limonium vulgare* and *Salicornia europaea* were  
402 typically among the most <sup>2</sup>H-enriched. The exception to this overall pattern among species  
403 occurred in March, when all species were characterized by δ<sup>2</sup>H values between -26‰ and -  
404 20‰ (Fig. 5; Supplementary Information Table 4). These extremely negative leaf water δ<sup>2</sup>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

409 Leaf water samples collected at 7:30-8:00 and 12:00-14:00 from *Elytrigia atherica*, *Atriplex*  
410 *portulacoides* and *Suaeda vera* at the ridge site allowed us to investigate diurnal shifts in leaf  
411 water isotopic composition. The C<sub>3</sub> grass *Elytrigia atherica* showed the greatest shift in the  
412  $\delta^2\text{H}$  of leaf water: it was 19‰ more positive at 12:00-14:00 than at 7:30-8:00. Leaf waters  
413 from two other plants showed a <sup>2</sup>H-enrichment of only 5-6‰ (Fig. 6; Supplementary  
414 Information Table 5).

415  
416 Statistical analysis (Minitab v.16, 2013) of interspecies variation in leaf water isotopic  
417 composition at each sampling interval indicated that leaf water <sup>2</sup>H/<sup>1</sup>H was not significantly  
418 different (Mann-Whitney U test, P>0.05, n= 8 for comparison of species growing from  
419 March to September 2012; n= 6 for comparison of species growing from May to September  
420 2012) among the Stiffkey species. However, *Phragmites australis*, the species that generally  
421 had the most <sup>2</sup>H-depleted leaf water isotopic signatures, was an exception. Leaf water from  
422 *Phragmites* was significantly different from the C<sub>4</sub> grass *Spartina anglica*, and the C<sub>3</sub> species  
423 *Salicornia europaea*, *Limonium vulgare*, and *Atriplex portulacoides* (student's t-test, P<0.05,  
424 n=6 individuals per species), but could not be distinguished statistically from leaf water from  
425 the other C<sub>3</sub> monocot *Elytrigia atherica*.

426

#### 427 **4.4 *n*-Alkane <sup>2</sup>H/<sup>1</sup>H composition**

428

429 Analysis of molecular distributions of *n*-alkanes from the sampled species (Supplementary  
430 Information Table 1 and 2) showed that *n*-C<sub>27</sub> and *n*-C<sub>29</sub> alkanes were the most abundant  
431 across all species. Because *n*-C<sub>27</sub> and *n*-C<sub>29</sub> alkane  $\delta^2\text{H}$  values were strongly correlated across  
432 the growing season (Fig. 2 in the Supplementary Information), we focused only on *n*-C<sub>29</sub>  $\delta^2\text{H}$   
433 values in all subsequent data analysis. The mean *n*-C<sub>29</sub>  $\delta^2\text{H}$  values from June 2011 showed a

434 total interspecies variation of 98‰, with the C<sub>3</sub> grass *Elytrigia atherica* having the most <sup>2</sup>H-  
435 depleted *n*-C<sub>29</sub> value and *Suaeda vera* the most <sup>2</sup>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<sub>29</sub> <sup>2</sup>H/<sup>1</sup>H) (*Suaeda vera*) to  
438 9‰ (*Atriplex portulacoides*). The greatest interspecies range in δ<sup>2</sup>H<sub>*n*-C<sub>29</sub></sub> was observed at the  
439 ridge site (93‰), while the lowest occurred in the upper marsh (24‰). *n*-C<sub>29</sub> from C<sub>3</sub> grasses  
440 was on average 45‰ more <sup>2</sup>H-depleted than that from the C<sub>4</sub> *Spartina anglica*. Overall, we  
441 observed the following pattern for *n*-C<sub>29</sub> alkane δ<sup>2</sup>H values: succulents > perennial herbs >  
442 evergreen shrubs > C<sub>4</sub> grass > C<sub>3</sub> monocots (Fig. 4; Supplementary Information Table 3).

443

444 The mean δ<sup>2</sup>H values of *n*-C<sub>29</sub> 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 <sup>2</sup>H/<sup>1</sup>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<sub>29</sub> 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<sup>2</sup>H values. However, unlike in  
456 the leaf water – where *Phragmites* was generally more negative than *Elytrigia* – the *n*-C<sub>29</sub>  
457 alkane δ<sup>2</sup>H values of *Elytrigia* were between 23 and 54‰ more negative than those of  
458 *Phragmites* across the entire growing season. In addition, the most <sup>2</sup>H-enriched *n*-C<sub>29</sub> values

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

465

466 Statistical analysis of interspecies variation in leaf wax hydrogen isotope compositions  
467 among all sampled species across the study period (Minitab v.16, 2013) revealed that the  
468  $^2\text{H}/^1\text{H}$  values of waxes were significantly different among most species (Mann-Whitney U  
469 test,  $P < 0.05$ ,  $n = 8$  for species growing from March to September 2012;  $n = 6$  for species  
470 growing from May to September 2012). Notable exceptions include a) *Suaeda vera*, and  
471 *Limonium vulgare*, and b) the two succulents *Suaeda vera* and *Salicornia europaea*.

472

#### 473 **4.5 $^2\text{H}/^1\text{H}$ fractionation between soil, xylem and leaf water and *n*-C<sub>29</sub> alkane**

474

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

479

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

484

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

488

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

489

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

493

494 In June 2011, the total variation in  $\epsilon$  between  $n\text{-C}_{29}$  and leaf water exceeded 100‰ (Fig. 7a &  
495 7b). Similar differences were identified throughout the 2012 growing season when the total  
496 variation in  $\epsilon_{\text{wax/lw}}$  exceeded 86‰ for all sampling intervals (Fig. 8). The greatest range in  
497  $\epsilon_{\text{wax/lw}}$  during the growing season was observed in August (109‰), and the lowest in  
498 September (86‰). The  $\text{C}_3$  grass *Elytrigia atherica* consistently had the lowest  $\epsilon_{\text{wax/lw}}$  value (-  
499 184 to -229‰), whilst *Suaeda vera* and *Limonium vulgare* recorded the highest (-79 to -  
500 144‰). Across all species, there was a general trend for  $\epsilon_{\text{wax/lw}}$  to become lower as the  
501 growing season progresses (Fig. 8; Supplementary Information Table 4). The variation in  
502 fractionation factors calculated for the plant species at Stiffkey is the largest range in  $\epsilon_{\text{wax/lw}}$   
503 reported to date for saltmarsh environments (c.f. Romero and Feakins, 2011).  $\epsilon_{\text{wax/sw}}$  values

504 for species growing at the three sites in 2012 ranged from -64‰ for *Salicornia* in March to -  
505 228‰ for *Elytrigia atherica* in September.  $\epsilon_{\text{wax/sw}}$  variability among the different plant  
506 species exceeded 89‰ throughout the growing season (Supplementary Information Table 4).

507

508 The C<sub>4</sub> grass, *Spartina anglica*, has  $\epsilon_{\text{wax/lw}}$  values that are higher (by up to 74 ‰) than those  
509 observed for the C<sub>3</sub> grass *Elytrigia atherica*. When the *Spartina* data are compared with other  
510 C<sub>3</sub> species collected in March and May 2012,  $\epsilon_{\text{wax/lw}}$  for *Spartina* is only 5-6‰ higher than in  
511 *Atriplex*, although it is 15-36‰ lower than the apparent fractionation observed in *Suaeda* and  
512 *Limonium*. As the growth season progresses, the difference in  $\epsilon_{\text{wax/lw}}$  among these species  
513 increases: in August, where the maximum variation is observed, the <sup>2</sup>H/<sup>1</sup>H fractionation  
514 between leaf water and leaf wax *n*-C<sub>29</sub> in *Spartina* is between 25 and 53‰ lower than these  
515 other C<sub>3</sub> shrubs and herbs (Supplementary Information Table 2).

516

517

## 5. DISCUSSION

518

519 Many previous studies have sought to explain variation in *n*-alkane <sup>2</sup>H/<sup>1</sup>H composition  
520 among different plant species by reference to the physical processes that control the  
521 movement of water molecules inside, outside and within the leaf. If we therefore, assume  
522 that interspecies variation in our leaf wax lipid  $\delta^2\text{H}$  is primarily driven by differences in the  
523 isotopic composition of leaf water, it follows that the > 100‰ range in *n*-alkane <sup>2</sup>H/<sup>1</sup>H  
524 compositions observed should be accounted for by a series of scenarios which affect leaf  
525 water  $\delta^2\text{H}$ . These mechanisms include: (i) differences in the isotopic composition of soil  
526 water among the three marsh sub-environments; (ii) differences in the isotopic composition  
527 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  $\delta^2\text{H}_{n\text{-C}29}$  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  $^2\text{H}$ -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  $\epsilon_{\text{wax/lw}}$  values observed within each sub-environment at Stiffkey (LM, R,  
554 UM) in June 2011 (Fig. 7a) suggest that source water isotopic composition is not a major  
555 factor controlling the hydrogen isotope signals preserved in the  $n\text{-C}_{29}$  alkane. This is  
556 supported by the limited variation observed in leaf water and  $n$ -alkane samples from selected  
557 plant species sampled in June 2011. Although no soil waters were collected in June, sampling  
558 of species growing in more than one location at Stiffkey allows for evaluation of the impact  
559 of marsh sub-environment on the  $^2\text{H}/^1\text{H}$  composition of leaf waters and leaf wax lipids. In  
560 theory, if spatial variation in environmental water across these sub-environments is  
561 significant, we would expect samples of the same individual species from multiple sites to  
562 have different  $\delta^2\text{H}$  leaf water and  $n$ -alkane compositions. Minor discrepancies in leaf water  
563 are observed in each species depending upon the particular sub-environment, for example  
564 13‰ between *Atriplex portulacoides* at the R and UM sites and 10‰ between *Triglochin*  
565 *maritima* at the LM and UM sites; Fig. 4). However, the magnitude of this spatial variability  
566 is insignificant when compared with the range of interspecies  $\delta^2\text{H}_{\text{lw}}$  values observed across  
567 the marsh as a whole (29‰). Differences in mean  $\delta^2\text{H}_{n\text{-C}_{29}}$  values for these species also show  
568 insignificant variation depending on sampling site – *Limonium*, *Triglochin* and *Suaeda* all  
569 vary by less than 5‰ between the LM and UM, while *Atriplex* shifts isotopically by 12‰  
570 (Fig. 4; SI Table 3). Again, the magnitude of these site-specific isotopic differences in  
571 individual species is negligible when compared with the ~100‰ interspecies variation in  
572  $\delta^2\text{H}_{n\text{-C}_{29}}$  among all sampled plants. In addition,  $\epsilon_{\text{wax/lw}}$  values from *Suaeda*, and *Limonium*  
573 show remarkable consistency across multiple sampling sites, with the maximum site-specific  
574 variation in one species (10‰ in *Triglochin*; 11‰ in *Atriplex*) an order of magnitude less  
575 than the total range in  $\epsilon_{\text{wax/lw}}$  observed in the data set as a whole (Fig. 7a, and 7b). We

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

579

## 580 **5.2 The significance of temporal differences in soil water**

581

582 In order to examine the influence of environmental water fully, it is important to consider  
583 whether differences in plant growth strategy expose them to seasonal variation in the source  
584 water  $\delta^2\text{H}$  signal. There is conflict in previous research over whether the *n*-alkane  $^2\text{H}/^1\text{H}$  is  
585 “locked in” at the beginning of the growing season or continually shifts in response to  
586 environmental or biological stimuli. Sachse et al. (2010) concluded that the *n*-alkane  $\delta^2\text{H}$   
587 values for field-grown barley were fixed early during the growing season and did not show  
588 seasonal shifts as the plants matured. A similar conclusion was reached by Tipple et al.  
589 (2013), who analysed the  $^2\text{H}/^1\text{H}$  composition of *n*-alkanes, stem water, and leaf water from  
590 the riparian angiosperm *Populus angustifolia* throughout a growing season. Leaf water values  
591 showed considerable seasonal variation of 55‰, however, *n*-alkane  $\delta^2\text{H}$  values remained  
592 relatively consistent in the mature leaf. This was interpreted to reflect the fixing of the *n*-  
593 alkane  $\delta^2\text{H}$  signal during the bud break period, where new waxes are produced from water  
594 and stored sugars, suggesting that the *n*-alkane  $^2\text{H}/^1\text{H}$  composition reflected these mixed  
595 biosynthate sources rather than providing an integrated signal of the growing season as a  
596 whole. In contrast, other studies propose that leaf waxes turnover continuously. Jetter and  
597 Schäffer (2006) considered that wax production was dynamic, with turnover and recycling of  
598 dominant compound classes during leaf development, whilst Gao et al. (2012) quantified  
599 regeneration rates of leaf wax compounds by the application of labelled irrigation water and  
600 concluded that *n*-C<sub>27</sub> – *n*-C<sub>31</sub> *n*-alkanes are replaced over a timescale of 71-128 days.

601

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

613

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

622

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

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  $\delta^2\text{H}$  from the lower marsh shows a considerably more  
631  $^2\text{H}$  -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  $\delta^2\text{H}$  of precipitation in North Norfolk in March is c. -62‰ (OIPC), and assuming a  
635 seawater  $\delta^2\text{H}$  value of 0‰, we calculate that rainfall contributed ~40% of the  $^2\text{H}/^1\text{H}$  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  $\delta^2\text{H}$  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<sub>29</sub> alkane  $\delta^2\text{H}$  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  $^2\text{H}$ -depleted soil water is found at this site (SI  
645 Table 4). Examination of mean monthly interpolated  $\delta^2\text{H}$  values of meteoric water at the  
646 Stiffkey site (OIPC; Supplementary Information Table 6) for our sampling periods show,  
647 however, modelled precipitation  $^2\text{H}/^1\text{H}$  ranges from -62‰ (March) to -48‰ (September).  
648 Soil waters from the ridge are consistently more  $^2\text{H}$ -enriched than these meteoric  
649 precipitation  $\delta^2\text{H}$  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  $^2\text{H}$ -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  $^2\text{H}$ -enriched seawater towards the soil surface (Plaster, 2009).  
655 When we consider these temporal shifts in environmental water  $^2\text{H}/^1\text{H}$  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  $\delta^2\text{H}_{n\text{-C}29}$  range among the ridge  
659 species. In our study, soil water  $\delta^2\text{H}$  varied by 31‰ at the ridge across the 2012 growth  
660 season, while the average interspecies range in  $\delta^2\text{H}_{n\text{-C}29}$  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 7<sup>th</sup> of September  
664 2012 allowed us to investigate diurnal changes in soil water  $\delta^2\text{H}$ . Sachse et al. (2010)  
665 suggested that one reason a direct 1:1 relationship was not observed between the  $\delta^2\text{H}$  of  
666 midday leaf water and  $\delta^2\text{H}_{n\text{-C}29}$  in barley was that plants were synthesising these compounds  
667 from water that had not been subjected to diurnal  $^2\text{H}$ -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  $\delta^2\text{H}_{n\text{-C}29}$  observed in the  
672 coastal plants at Stiffkey.

673

### 674 **5.3 The significance of soil water uptake by halophytes and non-halophytes**

675

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

686

687 Xylem waters collected between 12:00 and 14:00 at Stiffkey on the 7<sup>th</sup> of September 2012  
688 allow us to consider whether interspecies variation in fractionation occurring during water  
689 uptake ( $\Delta^2\text{H}$ ) can explain the variation in  $\delta^2\text{H}_{n\text{-C}29}$  in our data set.  $\Delta^2\text{H}$  values for the Stiffkey  
690 halophytes (those species with an Ellenberg value in excess of 4) show a much greater range  
691 than that published by Ellsworth and Williams (2007); however the maximum fractionation  
692 observed for *Atriplex* is still only 28‰, compared with a minimum fractionation of 4‰ in  
693 *Limonium vulgare*. This variation in fractionation during water uptake does not explain the  
694 41‰ difference between their  $\delta^2\text{H}_{n\text{-C}29}$  values. Equally, *Atriplex* and *Suaeda* growing on the  
695 ridge have the same  $\Delta^2\text{H}$  values (28‰), but their  $\delta^2\text{H}_{n\text{-C}29}$  values differ by 25‰

696

697 Some species at Stiffkey are merely salt tolerant and not classified as true halophytes. These  
698 include the common reed *Phragmites australis* (Hill et al., 1999; Mauchamp and Mésleard,  
699 2001) and *Elytrigia atherica* (Hill et al., 1999). Interestingly, these species also show xylem  
700 water values more negative than the soil water at their sampling location at the ridge site (Fig.

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

708

#### 709 **5.4 The significance of leaf water**

710

711 Physical differences among plants with different life forms, leading to various patterns of  
712 utilization of environmental water, have been used to explain variation in  $\delta^2\text{H}$  *n*-alkane values  
713 observed between both woody plants and grass (Liu et al., 2006). For instance, morphological  
714 characteristics have been identified as factors exerting a strong influence upon leaf water  
715 isotopic <sup>18</sup>O-enrichment (Helliker and Ehleringer, 2002; Barbour et al., 2004). Kahmen *et al.*  
716 (2008) suggested that leaf water isotopic <sup>18</sup>O-enrichment can differ even among species that  
717 are closely related because of differences in the “effective path length” (the distance that  
718 water is required to flow from source to evaporation site) in their leaves, which would  
719 influence the flow of isotopically enriched water back from the sub-stomatal cavity. Similar  
720 factors could potentially influence hydrogen isotopic composition of leaf water as well.

721

722 Studies seeking to apply factors relating to leaf water <sup>2</sup>H-enrichment to *n*-alkane data have  
723 attempted to explain observed variation in *n*-alkane <sup>2</sup>H/<sup>1</sup>H in terms of differences in plant life  
724 form on the basis that these physical differences could have influenced evapotranspiration of  
725 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  $\delta^2\text{H}$  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  $\delta^2\text{H}_{\text{lw}}$  values range from +5‰ to +21‰ whilst  $\delta^2\text{H}_{n\text{-C}_{29}}$  values differ by over  
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  $^2\text{H}/^1\text{H}$  between these two plants  
740 only ranges from 6 to 12‰ between May and September 2012. Leaf wax  $n\text{-C}_{29}$   $^2\text{H}/^1\text{H}$  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  $\delta^2\text{H}$   
744 composition is three times lower than that observed in  $\delta^2\text{H}_{n\text{-C}_{29}}$  in June 2011, and consistently  
745 4-5 times lower throughout the seasonal time series from 2012.  $^2\text{H}/^1\text{H}$  composition of  $n$ -  
746 alkanes ( $\delta^2\text{H}_{n\text{-C}_{29}}$ ) 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 ( $\delta^2\text{H}_{\text{lw}}$ ) show a total variation of only 29‰ (Supplementary Information Table 4). This  
749 contrast between a large variability of  $n$ -alkane  $\delta^2\text{H}$  and a small range of leaf water  $\delta^2\text{H}$   
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<sub>29</sub> alkane show 103‰ variation among sampled species, with  
752 only 6‰ shifts in leaf water, whilst in August 2012 the  $n$ -C<sub>29</sub> range exceeds 120‰ and leaf  
753 waters vary by only 29‰. *Phragmites australis* generally has the most negative leaf water  
754 <sup>2</sup>H/<sup>1</sup>H profile, whilst *Limonium vulgare*, *Spartina anglica* and *Salicornia* have leaf waters  
755 that are all generally <sup>2</sup>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 <sup>2</sup>H/<sup>1</sup>H among each species  
758 shows that March 2012 is significantly different from all other months. The range in leaf  
759 water  $\delta^2\text{H}$  in March 2012 is quite limited compared with all other sampling periods. Even if  
760 the  $n$ -alkane <sup>2</sup>H/<sup>1</sup>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  $\delta^2\text{H}_{n\text{-C}_{29}}$  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 <sup>2</sup>H-depletion and <sup>2</sup>H-enrichment in leaf water and  $n$ -C<sub>29</sub>  
766 alkane values do not co-vary, i.e. any similarity in leaf water <sup>2</sup>H/<sup>1</sup>H composition does not  
767 necessarily lead to a similarity in  $n$ -alkane  $\delta^2\text{H}$  values. Figure 2 presents data from the  
768 September 2012 sampling period, and shows that for species with very similar leaf water  
769 <sup>2</sup>H/<sup>1</sup>H compositions,  $n$ -alkane values can vary considerably. For example, whilst *Limonium*  
770 *vulgare* and *Salicornia* have the most <sup>2</sup>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  $\delta^2\text{H}_{\text{lw}}$  of *Limonium* and *Elytrigia* is 19‰, while the range in  $n$ -C<sub>29</sub> between these species  $\delta^2\text{H}$   
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<sub>29</sub> 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 <sup>2</sup>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 <sup>2</sup>H-depleted  $n$ -C<sub>29</sub> alkane value. Similarly,  
782 the C<sub>4</sub> grass *Spartina anglica* has the most <sup>2</sup>H-depleted  $n$ -C<sub>29</sub> alkane (-156‰) value in the low  
783 marsh, but one of the more <sup>2</sup>H-enriched leaf waters (+27‰). This lack of correlation between  
784 leaf water and leaf wax  $\delta^2\text{H}$  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<sub>29</sub> alkanes that differ by as  
787 much as 20‰ (Fig. 4).

788

789 At the ridge, where the greatest range in  $\epsilon_{\text{wax/lw}}$  values is observed in June 2011, this lack of  
790 correlation between leaf water and  $n$ -C<sub>29</sub> alkane <sup>2</sup>H/<sup>1</sup>H composition is also present (Fig. 4).  
791 Here, it is the C<sub>3</sub> reed, *Phragmites australis* that has the most <sup>2</sup>H-depleted leaf water (+5‰),  
792 but the  $n$ -C<sub>29</sub>  $n$ -alkane  $\delta^2\text{H}$  value for this species does not follow this trend (Fig. 4). The most  
793 <sup>2</sup>H-depleted  $n$ -C<sub>29</sub> alkane value on the ridge is in fact found in another C<sub>3</sub> grass, *Elytrigia*  
794 *atherica*, which has a leaf water  $\delta^2\text{H}$  value of +15‰. As observed in the low marsh, similar  
795 leaf water  $\delta\text{D}$  values do not result in similar  $n$ -C<sub>29</sub> alkane  $\delta^2\text{H}$  values: *Atriplex portulacoides*,  
796 and *Suaeda vera* and *Elytrigia atherica* all record leaf water <sup>2</sup>H/<sup>1</sup>H values ranging from +15  
797 to +21‰, but differ by 93‰ in terms of their  $n$ -C<sub>29</sub> alkane  $\delta^2\text{H}$  values. Even in the upper  
798 marsh, where the  $\delta^2\text{H}$  values display the smallest overall range among plant species,  
799 *Triglochin maritima* and *Atriplex portulacoides* record the highest leaf water  $\delta^2\text{H}$  values but  
800 in contrast have lowest  $n$ -C<sub>29</sub> alkane  $\delta^2\text{H}$  values (Fig 4). Statistical analysis of interspecies

801 variation in  $n$ -C<sub>29</sub> hydrogen isotope composition supports our finding that leaf water <sup>2</sup>H/<sup>1</sup>H is  
802 of limited relative importance in controlling leaf wax  $\delta^2$ H values. Variation in midday leaf  
803 water  $\delta^2$ H among the sampled species was not found to be statistically significant, while in  
804 contrast interspecies variation in  $n$ -C<sub>29</sub>  $\delta^2$ H was, suggesting some other mechanism was  
805 responsible for the >100‰ range in  $n$ -C<sub>29</sub> we report.

806

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

820

## 821 **5.5 Comparison of <sup>2</sup>H/<sup>1</sup>H fractionation among C<sub>3</sub> and C<sub>4</sub> plants at Stiffkey with** 822 **previously published research**

823

824 Earlier work has suggested that C<sub>3</sub> vs. C<sub>4</sub> plants have relatively invariant fractionation factors  
825 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<sub>3</sub> (-117±27‰) and  
827 C<sub>4</sub> (-132±12‰) plants (Chikaraishi and Naraoka, 2003; Chikaraishi et al., 2004), which  
828 continue to be applied to modern vegetation studies (Tippie 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 <sup>2</sup>H/<sup>1</sup>H diversity - if, for example,  
831 fractionation is calculated between leaf water and the *n*-C<sub>29</sub> alkane for September 2012, only  
832 half of the C<sub>3</sub> plants sampled have  $\epsilon_{\text{wax/lw}}$  values that fall within the range predicted by  
833 Chikaraishi and Naraoka (2003; 2004; Supplementary Information Table 4). The remaining  
834 C<sub>3</sub> species, which include *Elytrigia atherica*, *Phragmites australis* and *Atriplex*  
835 *portulacoides*, have  $\epsilon_{\text{wax/water}}$  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<sub>3</sub> species conform to the predicted values (Fig. 7b), while between March and August  
838 2012, only *Limonium vulgare*, *Suaeda vera* and *Salicornia europaea* have  $\epsilon_{\text{wax/lw}}$  values that  
839 regularly fall within the predicted -90 to -144‰ range for C<sub>3</sub> species (Chikaraishi and  
840 Naraoka, 2003; 2004). With regards to the C<sub>4</sub>-plant group, our calculated  $\epsilon_{\text{wax/lw}}$  values for  
841 the C<sub>4</sub> 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<sub>4</sub> species  
843 published by Chikaraishi and Naraoka (2003, 2004; Fig. 7b; Fig. 8).

844

845 A consistent difference in apparent fractionation among C<sub>3</sub> and C<sub>4</sub> species has also been  
846 identified in some studies. For example, Chikaraishi and Naraoka (2003) presented data  
847 suggesting that C<sub>4</sub> species had higher apparent fractionation factors compared with C<sub>3</sub>  
848 angiosperms and gymnosperms. However, plant functional types were not distinguished in

849 this study, and large standard deviations for the mean  $\epsilon_{\text{wax/w}}$  values ( $C_3 = -116 \pm 25\%$ ,  $C_4 = -$   
850  $133 \pm 12\%$ ) 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  $^2\text{H}/^1\text{H}$  compositions  
852 of  $-150.4 \pm 42.6\%$ , while that  $n$ -alkane  $\delta^2\text{H}$  signatures in  $C_3$  species average  $-175.7 \pm 29.5\%$ .  
853 Smith and Freeman (2006) limited their study to  $C_3$  and  $C_4$  grasses, and found that  $\epsilon$  values  
854 were  $\sim 20\%$  more negative in  $C_3$  grasses relative to  $C_4$  grasses, resulting in more negative  $n$ -  
855 alkane  $^2\text{H}/^1\text{H}$  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  $\delta^2\text{H}$  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  $\epsilon_{\text{wax/lw}}$  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  $-154 \pm 29\%$ . 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  $^2\text{H}/^1\text{H}$  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  $^2\text{H}/^1\text{H}$  of the *n*-C<sub>29</sub> alkane within a range of  
881 halophytic and non-halophytic C<sub>3</sub> and C<sub>4</sub> 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  $\delta^2\text{H}_{n\text{-C}_{29}}$  data, and the 150‰ range in  $\epsilon_{\text{wax/lw}}$  values, extend beyond the typical values for C<sub>3</sub>  
885 and C<sub>4</sub> 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  $\delta^2\text{H}$  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  $^2\text{H}/^1\text{H}$  composition, and (ii) fractionation between source  
894 water and *n*-alkane  $^2\text{H}/^1\text{H}$ , 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  $^2\text{H}/^1\text{H}$ . However little is currently known about

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

909

910

911

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912

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1120

1121 **Figure captions**

1122

1123 **Figure 1:** Aerial photograph (scale *c.* 2.0 x 1.8 km) of Stiffkey marsh, North Norfolk, UK  
1124 showing the location of the three study sites. Note the presence of an intricate network of  
1125 inlet channels delivering seawater to low-lying areas adjacent to Site 3 in the upper marsh.  
1126 (Copyright: Cambridge University Collection of Air Photographs).

1127

1128 **Figure 2:** Measured soil water  $\delta^2\text{H}$  (black diamonds), xylem water  $\delta^2\text{H}$  (grey squares), leaf  
1129 water  $\delta^2\text{H}$  (white triangles), and *n*-alkane  $\delta^2\text{H}$  (circles) values from all species sampled in  
1130 September 2012. LV = *Limonium vulgare*, SE = *Salicornia europaea*, SV = *Suaeda vera*, SA  
1131 = *Spartina anglica*, AP = *Atriplex portulacoides*, PA = *Phragmites australis*, EA = *Elytrigia*  
1132 *atherica*. The standard error did not exceed 2‰ for soil, xylem, leaf waters and 9‰ for *n*-  
1133 alkane measurements.

1134

1135 **Figure 3:** Measured xylem water  $\delta^2\text{H}$  values for three species sampled at the ridge site  
1136 between 7:30 and 8:00 and again between 12:00 and 14:00 on 7<sup>th</sup> September 2012. The  
1137 maximum standard error associated with these measurements was 2‰.

1138

1139 **Figure 4:** Measured *n*-C<sub>29</sub> alkane  $\delta^2\text{H}$  (black circles) and leaf water  $\delta^2\text{H}$  (white circles) values  
1140 for all plants sampled across the Stiffkey marsh in June 2011 (“C3” and “C4” refer to plant  
1141 biochemical pathways). Predicted  $\delta^2\text{H}$  values of seawater (grey line) and precipitation (grey  
1142 shading) are also shown. Plants are grouped by sampling site (Low marsh, Ridge, Upper  
1143 marsh). Each data point represents a collection of greater than five leaves from a minimum of  
1144 three separate plants. Maximum standard error associated with these measurements was 5‰  
1145 for *n*-alkane values and 1‰ for leaf waters. The isotopic composition of sea water (0‰) is

1146 highlighted by the straight grey line, whilst the grey shaded area illustrates the maximum  
1147 seasonal range in precipitation  $^2\text{H}/^1\text{H}$  composition estimated using the Online Isotopes in  
1148 Precipitation Calculator (Bowen et al., 2005).

1149

1150 **Figure 5:** Seasonal variation in  $n\text{-C}_{29}$  alkane  $\delta^2\text{H}$  and leaf water  $\delta^2\text{H}$  values for all plants  
1151 sampled during the 2012 growth season. Each data point represents a collection of greater  
1152 than five leaves from a minimum of three separate plants. The maximum standard error  
1153 associated with these measurements was 8‰ for  $n\text{-C}_{29}$  alkane and 2‰ for leaf water.

1154

1155 **Figure 6:** Measured leaf water  $\delta^2\text{H}$  values for three species sampled at the ridge site between  
1156 7:30 and 8:00 and again between 12:00 and 14:00 on 7<sup>th</sup> September 2012. The maximum  
1157 standard error associated with these measurements was 2‰.

1158

1159 **Figure 7:** Calculated fractionation ( $\epsilon_{\text{wax/lw}}$  ‰) between  $n\text{-C}_{29}$  alkane  $\delta^2\text{H}$  and leaf water  $\delta^2\text{H}$   
1160 from samples collected in June 2011 at Stiffkey saltmarsh. Plants are grouped according to a)  
1161 sampling locations and b) the plant types.

1162

1163 **Figure 8:** Calculated fractionation ( $\epsilon_{\text{wax/lw}}$  ‰) between  $n\text{-C}_{29}$  alkane  $\delta^2\text{H}$  and leaf water  $\delta^2\text{H}$   
1164 from samples collected across the 2012 growth season at Stiffkey saltmarsh. SV = *Suaeda*  
1165 *vera*, LV = *Limonium vulgare*, SE = *Salicornia europaea*, AP = *Atriplex portulacoides*, SA =  
1166 *Spartina anglica*, PA = *Phragmites australis*, EA = *Elytrigia atherica*.

1167

1168 **SI Figure 1:**  $n$ -Alkane average chain length (ACL) values from May and September for all  
1169 species sampled across the 2012 growth season.

1170

1171 **SI Figure 2:** Bivariate plot of ACL and  $n$ -C<sub>29</sub> alkane  $\delta^2\text{H}$  (September 2012) showing no  
1172 correlation between the two parameters. Letters in parenthesis denote plant species: AP =  
1173 *Atriplex portulacoides*, EA = *Elytrigia atherica*, LV = *Limonium vulgare*, PA = *Phragmites*  
1174 *australis*, SE = *Salicornia europaea*, SA = *Spartina anglica*, SV = *Suaeda vera*.

1175

1176 **SI Figure 3:** Bivariate plot of  $n$ -C<sub>27</sub> and  $n$ -C<sub>29</sub> alkane  $\delta^2\text{H}$  values for all species sampled  
1177 across the 2012 growth season showing a strong correlation between the two sets of data.