

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 C_3 and 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 ^2H -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 C_3 and C_4 plant groups. The magnitude of variability in the $\delta^2\text{H}$
59 values of *n*-alkyl lipids among plant species growing at the same geographical location
60 suggests, however, that this assumption may not necessarily be valid. Interpretation of
61 sedimentary *n*-alkyl $\delta^2\text{H}$ data is further complicated by limited understanding of the reasons
62 for this large interspecies variability. Sachse et al. (2012) provided a comprehensive review

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 ^2H -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 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 $\delta^2\text{H}$ values. However, their model, which assumed a 1:1 relationship between leaf
101 water (source) and leaf wax (product), overestimated ^2H -enrichment of the *n*- C_{31} alkane. The
102 authors proposed that this discrepancy could be due to a ^2H -depleted pool of water used
103 during biosynthesis, which may have originated from spatial inhomogeneity in ^2H -
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 ^2H -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 ^2H -enrichment was species-specific; with 18 to 68% of the leaf
113 water ^2H -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 ^2H -
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 ^2H -depletion in lipid $^2\text{H}/^1\text{H}$ observed during the
121 summer months – contrary to the anticipated ^2H -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 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 C_3 and 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

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 C₄ grass *Spartina anglica*, C₃ annuals *Salicornia europaea* and *Limonium*
206 *vulgare*, and occasionally the C₃ shrub *Atriplex portulacoides*. The gravel ridge supports a
207 range of C₃ 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*

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 ≤ 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 H_2
304 reference gas $\delta^2\text{H}$ values after 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⁻¹, and then at 8 °C
328 min⁻¹ to 320 °C (10 min). *n*-Alkanes were identified by comparison of their elution times with
329 *n*-C₁₆ to *n*-C₃₀ alkane standard (A. Schimmelmann, Indiana University). Average chain length
330 (ACL) and carbon preference index (CPI; Supplementary Information Tables 1 and 2) values
331 were calculated following the approach of Zhang et al., (2006).

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⁻¹ to 220
339 °C. A second temperature ramp to a final temperature of 320 °C at a rate of 6 °C min⁻¹
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 (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*-C₁₆ to *n*-C₃₀ 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 H_2 reference gas $\delta^2\text{H}$ values after H_3^+ correction was $\pm 6\%$.
347 Typical absolute differences in *n*-C₂₉ 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 ^2H -depleted in March (-27‰) and most ^2H -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‰ ²H-depleted compared with samples
366 collected between 12:00-14:00 (Supplementary Information Table 5).

367

368 **4.2 Xylem water ²H/¹H 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 ²H/¹H 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 ²H/¹H 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 ²H-enriched than those sampled from other locations, but the
387 range of variation was low compared to gross interspecies differences: $\delta^2\text{H}_{\text{LW}}$ from *Atriplex*
388 *Portulacoides* varied by 13‰ across the marsh, with the most ²H-depleted leaf water found at
389 the ridge site and the most ²H-enriched in the upper marsh; $\delta^2\text{H}_{\text{LW}}$ 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 ²H-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 ²H-depleted values (-26‰, *Limonium vulgare*, March) and the most
397 ²H-enriched (+20‰, *Salicornia europaea*, September). Species-specific variation in leaf
398 water $\delta^2\text{H}$ was most limited in March (6‰) and greatest in August (29‰). Leaf waters from
399 all species were generally most ²H-depleted in March, and ²H-enriched in September.
400 *Elytrigia atherica* and *Phragmites australis* were generally the most ²H-depleted in terms of
401 leaf water $\delta^2\text{H}$, whilst *Spartina anglica*, *Limonium vulgare* and *Salicornia europaea* were
402 typically among the most ²H-enriched. The exception to this overall pattern among species
403 occurred in March, when all species were characterized by $\delta^2\text{H}$ values between -26‰ and -
404 20‰ (Fig. 5; Supplementary Information Table 4). These extremely negative leaf water $\delta^2\text{H}$
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 C₃ 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 ²H-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 ²H/¹H 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 ²H-depleted leaf water isotopic signatures, was an exception. Leaf water from
422 *Phragmites* was significantly different from the C₄ grass *Spartina anglica*, and the C₃ 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 C₃ monocot *Elytrigia atherica*.

426

427 **4.4 *n*-Alkane ²H/¹H composition**

428

429 Analysis of molecular distributions of *n*-alkanes from the sampled species (Supplementary
430 Information Table 1 and 2) showed that *n*-C₂₇ and *n*-C₂₉ alkanes were the most abundant
431 across all species. Because *n*-C₂₇ and *n*-C₂₉ 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*-C₂₉ $\delta^2\text{H}$
433 values in all subsequent data analysis. The mean *n*-C₂₉ $\delta^2\text{H}$ values from June 2011 showed a

434 total interspecies variation of 98‰, with the C₃ grass *Elytrigia atherica* having the most ²H-
435 depleted *n*-C₂₉ value and *Suaeda vera* the most ²H-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*-C₂₉ ²H/¹H) (*Suaeda vera*) to
438 9‰ (*Atriplex portulacoides*). The greatest interspecies range in δ²H_{*n*-C₂₉} was observed at the
439 ridge site (93‰), while the lowest occurred in the upper marsh (24‰). *n*-C₂₉ from C₃ grasses
440 was on average 45‰ more ²H-depleted than that from the C₄ *Spartina anglica*. Overall, we
441 observed the following pattern for *n*-C₂₉ alkane δ²H values: succulents > perennial herbs >
442 evergreen shrubs > C₄ grass > C₃ monocots (Fig. 4; Supplementary Information Table 3).

443

444 The mean δ²H values of *n*-C₂₉ 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 ²H/¹H 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*-C₂₉ 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 δ²H values. However, unlike in
456 the leaf water – where *Phragmites* was generally more negative than *Elytrigia* – the *n*-C₂₉
457 alkane δ²H values of *Elytrigia* were between 23 and 54‰ more negative than those of
458 *Phragmites* across the entire growing season. In addition, the most ²H-enriched *n*-C₂₉ 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 $n\text{-C}_{29}$ alkane $\delta^2\text{H}$ values across all sampling intervals (Fig. 5). Cross-plotting the $n\text{-C}_{29}$ 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 $n\text{-C}_{29}$ 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). ^2H -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_4 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 ϵ 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 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 C₄ grass, *Spartina anglica*, has $\epsilon_{\text{wax/lw}}$ values that are higher (by up to 74 ‰) than those
509 observed for the C₃ grass *Elytrigia atherica*. When the *Spartina* data are compared with other
510 C₃ 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 ²H/¹H fractionation
514 between leaf water and leaf wax *n*-C₂₉ in *Spartina* is between 25 and 53‰ lower than these
515 other C₃ shrubs and herbs (Supplementary Information Table 2).

516

517

5. DISCUSSION

518

519 Many previous studies have sought to explain variation in *n*-alkane ²H/¹H 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 ²H/¹H
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 ^2H -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*-C₂₇ – *n*-C₃₁ *n*-alkanes are replaced over a timescale of 71-128 days.

601

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

613

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

622

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

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 ^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 $\delta^2\text{H}$ 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*-C₂₉ 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 ^2H -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 ^2H -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 ^2H -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 ^2H -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 7th 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 ^2H -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 ^2H -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 ^2H -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 7th 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 ²H-
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 ¹⁸O-enrichment (Helliker and Ehleringer, 2002; Barbour et al., 2004). Kahmen *et al.*
716 (2008) suggested that leaf water isotopic ¹⁸O-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 ²H-enrichment to *n*-alkane data have
723 attempted to explain observed variation in *n*-alkane ²H/¹H 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 -C₂₉ alkane show 103‰ variation among sampled species, with
752 only 6‰ shifts in leaf water, whilst in August 2012 the n -C₂₉ range exceeds 120‰ and leaf
753 waters vary by only 29‰. *Phragmites australis* generally has the most negative leaf water
754 ²H/¹H profile, whilst *Limonium vulgare*, *Spartina anglica* and *Salicornia* have leaf waters
755 that are all generally ²H-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 ²H/¹H 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 ²H/¹H 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 ²H-depletion and ²H-enrichment in leaf water and n -C₂₉
766 alkane values do not co-vary, i.e. any similarity in leaf water ²H/¹H 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 ²H/¹H compositions, n -alkane values can vary considerably. For example, whilst *Limonium*
770 *vulgare* and *Salicornia* have the most ²H-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 -C₂₉ 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 -C₂₉ 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 ²H-depleted leaf water value (+22‰) of plants found in this sub-environment, but this does
781 not result in *Triglochin maritima* having the most ²H-depleted n -C₂₉ alkane value. Similarly,
782 the C₄ grass *Spartina anglica* has the most ²H-depleted n -C₂₉ alkane (-156‰) value in the low
783 marsh, but one of the more ²H-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 -C₂₉ 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 -C₂₉ alkane ²H/¹H composition is also present (Fig. 4).
791 Here, it is the C₃ reed, *Phragmites australis* that has the most ²H-depleted leaf water (+5‰),
792 but the n -C₂₉ n -alkane $\delta^2\text{H}$ value for this species does not follow this trend (Fig. 4). The most
793 ²H-depleted n -C₂₉ alkane value on the ridge is in fact found in another C₃ 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 δD values do not result in similar n -C₂₉ alkane $\delta^2\text{H}$ values: *Atriplex portulacoides*,
796 and *Suaeda vera* and *Elytrigia atherica* all record leaf water ²H/¹H values ranging from +15
797 to +21‰, but differ by 93‰ in terms of their n -C₂₉ 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 -C₂₉ alkane $\delta^2\text{H}$ values (Fig 4). Statistical analysis of interspecies

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

820

821 **5.5 Comparison of ²H/¹H fractionation among C₃ and C₄ plants at Stiffkey with** 822 **previously published research**

823

824 Earlier work has suggested that C₃ vs. C₄ 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 C₃ (-117±27‰) and
827 C₄ (-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 ²H/¹H diversity - if, for example,
831 fractionation is calculated between leaf water and the *n*-C₂₉ alkane for September 2012, only
832 half of the C₃ 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 C₃ 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 C₃ 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 C₃ species (Chikaraishi and
840 Naraoka, 2003; 2004). With regards to the C₄-plant group, our calculated $\epsilon_{\text{wax/lw}}$ values for
841 the C₄ 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 C₄ species
843 published by Chikaraishi and Naraoka (2003, 2004; Fig. 7b; Fig. 8).

844

845 A consistent difference in apparent fractionation among C₃ and C₄ species has also been
846 identified in some studies. For example, Chikaraishi and Naraoka (2003) presented data
847 suggesting that C₄ species had higher apparent fractionation factors compared with C₃
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 ϵ 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*-C₂₉ alkane within a range of
881 halophytic and non-halophytic C₃ and C₄ 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 C₃
885 and C₄ 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 7th September 2012. The
1137 maximum standard error associated with these measurements was 2‰.

1138

1139 **Figure 4:** Measured *n*-C₂₉ 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 7th 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 -C₂₉ alkane $\delta^2\text{H}$ (September 2012) showing no
1172 correlation between the two parameters. Letters in parenthesis denote plant species: AP =
1173 *Atriplex portulacoides*, EA = *Elytrigia atherica*, LV = *Limonium vulgare*, PA = *Phragmites*
1174 *australis*, SE = *Salicornia europaea*, SA = *Spartina anglica*, SV = *Suaeda vera*.

1175

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