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1 **Plant and root-zone water isotopes are difficult to measure, explain, and predict: some**
2 **practical recommendations for determining plant water sources**

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16 **Abstract**

17 1) Stable isotope ratios of water ($\delta^{18}\text{O}$, $\delta^2\text{H}$) have long been used to study a core question in plant
18 ecology and ecohydrology: "From where do plants take up water?" Indeed, decades of research has
19 involved sampling potential plant water sources in the subsurface, classifying those sources as distinct
20 endmembers (e.g., deep versus shallow soil waters), and then evaluating their contributions to a
21 xylem water sample through mixing-model analysis to identify the depths of root water uptake.

22 2) However, more detailed interrogations of the subsurface and plant domains have revealed under-
23 considered transport and isotopic-fractionation phenomena. These now apparent complexities raise
24 new questions and challenge the many past assumptions inherent in endmember-mixing models that
25 now seem overly simple.

- 26 3) Here, we introduce discussions of these recent insights and provide an overview of isotope effects
27 that occur naturally in the root zone and in the plant, as well as artificially during sample handling.
28 Better accounting for these complexities and their associated uncertainties can lead to more accurate
29 and robust study designs, analytical frameworks, and, ultimately, inferences.
- 30 4) Finally, to more robustly characterize plant water sources using $\delta^{18}\text{O}$ and $\delta^2\text{H}$, we provide some
31 practical recommendations that aim at maximizing the isotopic contrast between endmembers
32 and/or minimizing potential uncertainties.

33

34 **Key-words:** ecohydrology; endmember-mixing models; plant water uptake; review; root zone; uncertainties;
35 stable water isotopes

36

37 1. Introduction and motivation

38 Plant water use can represent up to 90% of terrestrial evapotranspiration (Jasechko et al., 2013), and
39 therefore is an important driver of the global water cycle (Sellers et al., 1997). Because plant water relations
40 are closely linked to carbon and nutrient relations (Schulze, 1982), understanding plant-water supplies is also
41 key to predict global carbon and nutrient cycles (Lange, Kappen, & Schultze, 2012), and to scale plant functions
42 to the ecosystem- and land-surface levels (Feddes et al., 2001; Grossiord et al., 2017; Javaux, Couvreur,
43 Vanderborgh, & Vereecken, 2013).

44 Root morphology (e.g. root diameter, root branching, root suberization, root hairs, rooting depth) and
45 the ability of roots to adjust their structure and physiology to environmental factors are two major drivers of
46 plant water uptake (Jackson, Sperry, & Dawson, 2000). These multiple rooting properties have been poorly
47 assessed thus far compared to aboveground functions and structures of plants, in part because of the difficulty
48 to access and measure belowground compartments (Isaac & Anglaere, 2013). Destructive methods, such as
49 excavating the whole root system of plants, inform how roots occupy soils; however, knowledge about where
50 roots are located does not necessarily imply where water uptake occurs from (Ehleringer & Dawson, 1992).
51 Whereas excavation studies can be useful to understand plant-physiological variations across climates, species
52 and soils, they do not provide insights into how zones of active root water uptake vary in time and space, and
53 how they correspond with soil-water conditions and plant functions.

54 For nearly 50 years, the analysis of stable isotope ratios of water ($\delta^{18}\text{O}$, $\delta^2\text{H}$) has provided a powerful
 55 tool to study plant water uptake processes (e.g., Dawson & Ehleringer, 1998; Dawson & Pate, 1996; Ehleringer
 56 & Dawson, 1992; Flanagan, Ehleringer, & Marshall, 1992; Penna et al., 2018; Phillips & Gregg, 2003; Unkovich,
 57 2001; White, Cook, Lawrence, & Broecker, 1985; U. Zimmermann, Ehhalt, & Muennich, 1967 and many more).
 58 Isotope data of xylem and root-zone water are often used in linear endmember-mixing models, under the
 59 assumption that the isotopes represent conservative tracers with no fractionation occurring during water
 60 uptake by the roots (U. Zimmermann et al., 1967) so that the isotope ratios in xylem water reflects the mixture
 61 of water sources that supply functional roots.

62 The simplest case of a linear endmember-mixing model that uses one isotope ratio (e.g., $\delta^{18}\text{O}$) to
 63 differentiate between the relative contributions (f) of two sources 1 and 2 (e.g., deep and shallow soil water)
 64 to a mixture (e.g., plant xylem water) takes the form (Phillips & Gregg, 2001)

$$65 \quad \delta_{mix} = f_1 \cdot \delta_1 + f_2 \cdot \delta_2, \quad (1)$$

66 where

$$67 \quad 1 = f_1 + f_2 \quad (2)$$

68 Through combining equations (1) and (2), the relative fractions of source 1 in the mixture can be quantified:

$$69 \quad f_1 = \frac{\delta_{mix} - \delta_2}{\delta_1 - \delta_2} \quad (3)$$

70 An analytical solution for f_1 and f_2 can only be obtained if the number of sources is $n+1$, with n being the
 71 number of isotopic tracers; even when measurements of both $\delta^{18}\text{O}$ and $\delta^2\text{H}$ are available, they often cannot
 72 be treated as independent tracers because they strongly co-vary (Craig, 1961). In cases where a mixture
 73 contains more than two water sources but only one isotope tracer ($\delta^{18}\text{O}$ or $\delta^2\text{H}$) can be used, the bounds for
 74 each individual fraction f_1, f_2, \dots, f_{n+1} can be estimated statistically (e.g., IsoSource, Phillips & Gregg, 2003; SIAR,
 75 Parnell, Inger, Bearhop, & Jackson, 2010). Often, these multi-source mixing models have been implemented
 76 into a Bayesian framework to include prior information about soil properties, root distribution, climate etc.
 77 (Moore & Semmens, 2008; Rothfuss & Javaux, 2017).

78 Most importantly, these mixing-model approaches allow for quantifying the uncertainties in the
 79 relative source contributions f on the condition that the uncertainties of the individual endmembers are

80 known (these endmember uncertainties due to analytical errors and spatiotemporal heterogeneity will be
 81 discussed in more detail below). Phillips and Gregg (2001) present the analytical solution for calculating the
 82 standard error (SE) in f_1 for a two-source mixture (Eq. 1):

$$83 \quad SE_{f_1}^2 = \frac{1}{(\delta_1 - \delta_2)^2} \cdot [SE_{\delta_{mix}}^2 + f_1^2 \cdot SE_{\delta_1}^2 + (1 - f_1)^2 \cdot SE_{\delta_2}^2] \quad (4)$$

84 Additional methods for propagating the errors in endmember signatures into the uncertainty in f have been
 85 extensively reviewed by others (e.g., Evaristo, McDonnell, & Clemens, 2017; Rothfuss & Javaux, 2017; Wang,
 86 Lu, & Fu, 2019).

87 Using linear mixing models requires that the endmembers capture the sources of plant water and that
 88 those defined endmembers are fully isotopically distinct; thus, intensive sampling can be required. Usually this
 89 involves sampling the vertical isotopic profile of the root zone so that variations across depths are understood.
 90 Then, natural isotopic break points are selected so that the endmembers describing different subsurface water
 91 pools can be identified (e.g., <0.3 m versus >0.3 m deep soil water in Figure 1). Alternatively, instead of
 92 defining plant source water endmembers by depth, they can be defined with respect to seasonal precipitation,
 93 which can provide different insights into how root distributions interplay with infiltration patterns (e.g., Allen,
 94 Kirchner, Braun, Siegwolf, & Goldsmith, 2019; Ehleringer, Phillips, Schuster, & Sandquist, 1991). In any case,
 95 the endmembers must be carefully defined because this process often involves assuming a binary division of
 96 the subsurface when root-zone waters actually vary gradually and continuously. Indeed, intensive sampling of
 97 root-zone water often reveals isotopic heterogeneities within defined endmembers, which influences the
 98 errors, and thus the strength of inference obtained from endmember-mixing models (Goldsmith et al., 2019;
 99 Oerter, Siebert, Bowling, & Bowen, 2019).

100 Advances in experimental and analytical methods have revealed many challenges in sampling all
 101 potential plant water sources and defining non-overlapping endmembers. In recent years, the isotopic
 102 composition of plant root zones has been increasingly investigated across diverse settings (see reviews by
 103 Penna et al., 2018 and Sprenger, Leistert, Gimbel, & Weiler, 2016). Importantly, some studies have linked
 104 root-zone isotopic heterogeneities to small-scale heterogeneities in transport and mixing processes (Sprenger,
 105 Llorens, Cayuela, Gallart, & Latron, 2019), and suggested the occurrence of isotopic fractionation effects at
 106 mineral-water interfaces (Y. Lin & Horita, 2016) and root-water interfaces (Vargas, Schaffer, Li, & Sternberg,

107 2017). Whereas the effects of these and other phenomena should manifest in temporally and spatially
108 (laterally and vertically) variable root-zone isotope ratios, most practical field sampling designs will not allow
109 for the over-sampling required to fully characterize and account for those heterogeneities in mixing-model
110 analyses (Goldsmith et al., 2019). Thus, careful design, application, and interpretation of root-water uptake
111 studies is warranted.

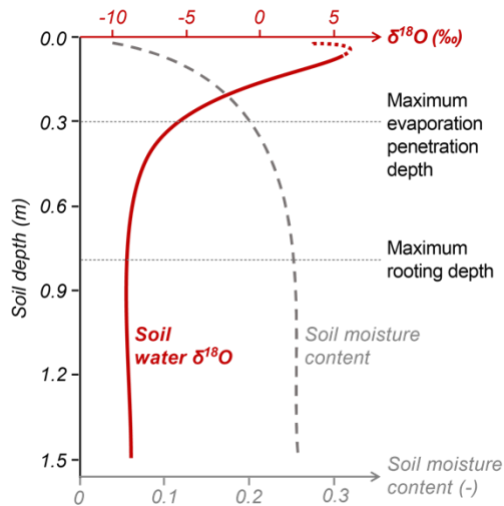
112 In this review, we show the diversity of isotope ratios that can naturally occur in the root zone and in
113 plants and how this diversity can influence the identification of potential plant source waters. We then discuss
114 these isotopic variations and the measurement and prediction challenges they convey in the context of
115 traditional mixing model analyses. Our ultimate objective is to offer practical guidance to facilitate inferring
116 the water sources used by plants through using stable water isotopes.

117

118 **2. Root-zone water: Characterizing and sampling across natural variations in** 119 **isotope endmembers**

120 Isotope endmembers of the root-zone water ($\delta_{root-zone}$) are typically characterized using one or
121 several isotope profiles of the subsurface, which are determined by extracting water from soils or other porous
122 media sampled at various depths. Conventionally, it has been assumed that these isotope profiles are near-
123 monotonic, with heavier isotope ratios in shallower depths and lighter isotope ratios in greater depths (solid
124 red line in Figure 1). This characteristic isotope-depth profile often occurs when shallow soil waters become
125 enriched as isotopically lighter water is preferentially evaporated (U. Zimmermann et al., 1967; Barnes &
126 Allison, 1984) or when isotopically heavier growing-season precipitation recharges the soils that supply
127 evapotranspiration (however, not always the most-recent precipitation is evapotranspired; Allen, von
128 Freyberg, Weiler, Goldsmith, & Kirchner, 2019). Beneath the maximum evaporation penetration depth,
129 infiltrated precipitation water mixes with previously stored water over seasons and years so that the isotopic
130 signature of soil water represents the long-term average of previous precipitation events that have recharged
131 these soils; thus, deeper soil waters are usually isotopically lighter than shallow soil waters.

Determining plant water sources with isotopes



132

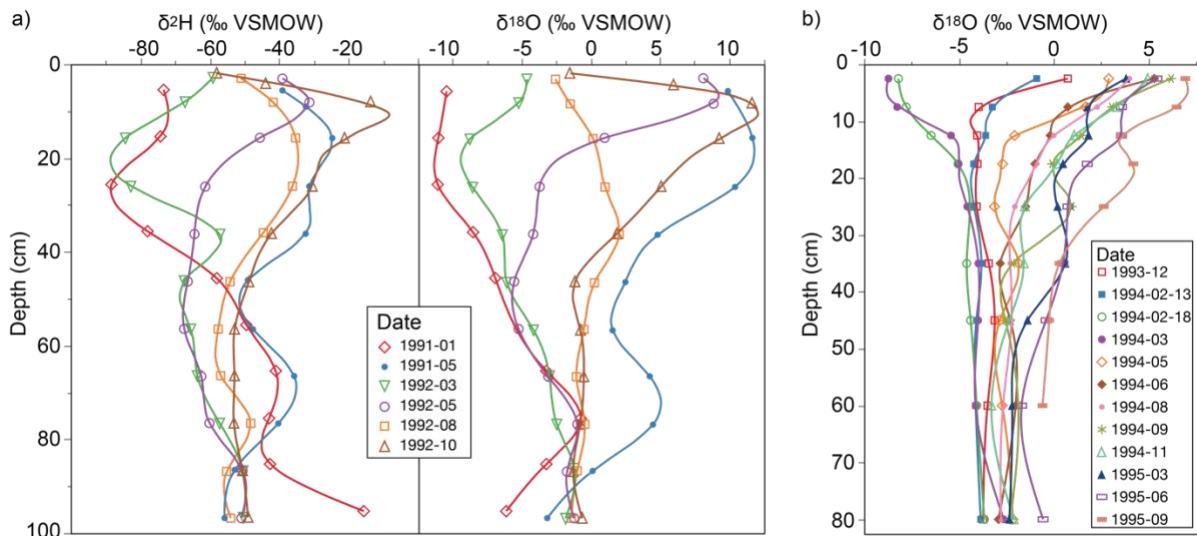
133 *Figure 1: Hypothetical example of soil moisture content (grey dashed line) and soil-water $\delta^{18}\text{O}$ (red solid line) for a sandy-*
134 *loam soil profile occupied by shallow-rooted plants. It is commonly assumed that the soil-water isotope profile is near-*
135 *monotonic with disproportionate amounts of the heavier isotopologues in the shallow layers mainly due to evaporative*
136 *fractionation. Isotopic inversions in the top few cm (red dashed line) result from fractionation associated with phase*
137 *changes of water within the profile. Figure re-drawn after Barnes and Allison (1983).*

138

139 2.1 Evaporation and transport result in lateral heterogeneities and non-monotonic isotope profiles

140 In most natural systems, $\delta_{\text{root-zone}}$ values do not monotonically decrease with depth if precipitation
141 inputs are isotopically variable (and they usually are; Dansgaard, 1964, Munksgaard, Wurster, Bass, & Bird,
142 2012) or if evaporatively enriched pore waters are transported downward. Such variations are likely to be
143 more extreme in regions with highly variable weather conditions; for instance, in a drying phase, the
144 evaporation penetration front near the soil surface will move progressively downward into the deeper
145 subsurface (Rothfuss et al., 2015). With precipitation, the (evaporatively enriched) isotope composition in the
146 upper depth profile may become attenuated or displaced downward such that the pre-event and post-event
147 isotope profiles differ (Sprenger et al., 2016). Figure 2a illustrates how dramatically $\delta_{\text{root-zone}}$ can vary across
148 a desert soil profile in Arizona, USA; isotope ratios in root-zone water up to 25cm depth seasonally vary by
149 more than 90‰ in $\delta^2\text{H}$ and 20‰ in $\delta^{18}\text{O}$. Even for wetter environments, such as on the humid Kohala
150 peninsula on Hawaii, the complex interplay between wetting and drying of the soil profile can result in up to
151 15‰ vertical variations in $\delta^{18}\text{O}$ (Figure 2b). The occurrence of these isotope fluctuations in the subsurface
152 imply that under-sampling certain depths could result in failing to identify the appropriate plant-water
153 sources.

Determining plant water sources with isotopes



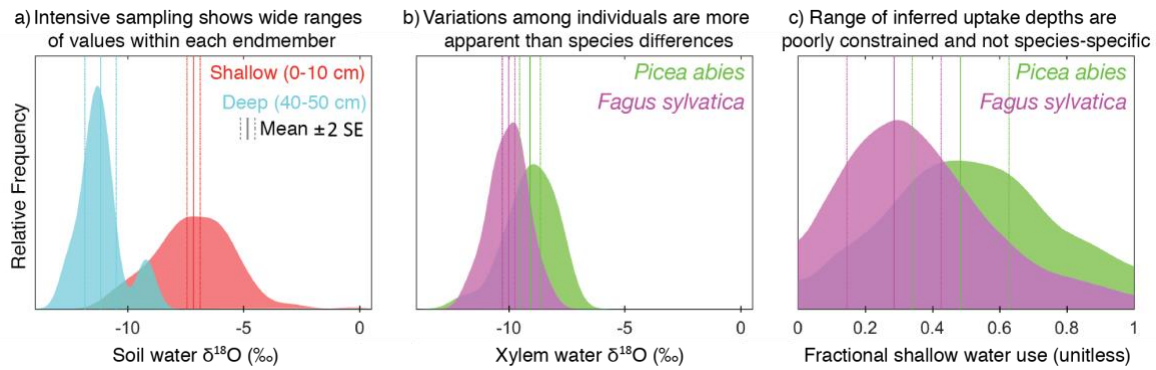
154

155 *Figure 2: a) Seasonal variations of $\delta^{18}\text{O}$ and $\delta^2\text{H}$ in soil water in a nearly bare desert soil (figure re-drawn from Liu, Phillips,*
 156 *Hoines, Campbell, & Sharma, 1995). b) $\delta^{18}\text{O}$ values in soil water under pasture grass in a weathered volcanic ash soil*
 157 *(Andisol) on the island of Hawaii. The climatic conditions were humid with a high variability in rainfall. Isotope data from*
 158 *two samples per campaign were averaged (original data from Table 4 in Hsieh, Chadwick, Kelly, & Savin, 1998).*

159 Beyond the vertical variability in $\delta_{\text{root-zone}}$ profiles, recent studies highlight substantial $\delta_{\text{root-zone}}$
 160 heterogeneity in the lateral dimension. Lateral isotopic variations have been attributed to subsurface
 161 properties, canopy interception and shading effects (e.g., Goldsmith et al., 2019; McCole & Stern, 2007). For
 162 instance, Figure 3a shows extremely heterogeneous shallow $\delta_{\text{root-zone}}$ across a 1-ha forest plot; isotope ratios
 163 were weakly spatially autocorrelated, and thus their variations could not be easily predicted without this level
 164 of extensive sampling (Goldsmith et al., 2019).

165 Where lateral heterogeneities in $\delta_{\text{root-zone}}$ are prominent, mixing-model analyses should consider
 166 the full range of individual potential endmember values instead of simply averaging across all $\delta_{\text{root-zone}}$
 167 values, which would dampen true variations and would yield a subsurface characterization that seems more
 168 well-mixed than probably ever actually exists (Figure 3a). When we calculate the relative source contributions
 169 f (Eq. 3) through including all potential endmember values we will obtain a range of solutions for each xylem
 170 water mixture (Figure 3c). Through combining each xylem water sample with each possible shallow-soil water
 171 endmember, one can see that the uncertainty in the two-component endmember mixing approach can be
 172 substantial, e.g., contributions of shallow soil water to *Picea abies* can vary from zero to 100% (Figure 3c).
 173 Thus, misinterpretations of endmember-mixing analyses are likely to happen if the heterogeneity of soil water
 174 (and xylem water) isotopic composition is not well characterized.

Determining plant water sources with isotopes



175

176 *Figure 3: $\delta^{18}\text{O}$ of soil water from two depths, $\delta^{18}\text{O}$ of xylem water from two species, and inferred use of shallow water from*
177 *mixing-model analysis, all from a single-day snapshot sampling across 1 ha of a forested hillslope in Switzerland (data are*
178 *from Goldsmith et al., 2019). All water samples (149 shallow soil samples, 8 deep soil samples, 22 *Picea abies* samples, and*
179 *35 *Fagus sylvatica* samples) were extracted by cryogenic vacuum distillation and then analyzed on a mass spectrometer;*
180 *detailed methods are described in the original Goldsmith et al. (2019) study. Here, we show these data to demonstrate that*
181 *intensive sampling can reveal wide ranges in isotope ratios within the endmember samples (panel a) and mixtures (b); these*
182 *ranges are often not revealed through typical sampling strategies, and means (\pm standard errors) can poorly capture the*
183 *distribution of actual potential values; the mixing-model standard errors were calculated using IsoError (Phillips & Gregg,*
184 *2001). Importantly (but not shown here), the shallow soil-water heterogeneity was not strongly structured, so any sample*
185 *could be associated with any tree; thus, the mixing-model analysis (c) shows the range of solutions for all trees for all*
186 *combinations of potential endmember values. While there are significant differences between the shallow and deep soil*
187 *water $\delta^{18}\text{O}$ mean values, or the two species xylem $\delta^{18}\text{O}$ water values, the distributions in panel (c) strongly overlap. While*
188 *the mixing-model analysis suggests that *P. abies* used shallower water than *F. sylvatica*, the ranges exceed 0-1 in both*
189 *(indeterminate values were cropped from the figure) and thus no clear characteristic depth of uptake could be inferred for*
190 *each species. Goldsmith et al.'s findings demonstrate that using mean values in the model could result in a*
191 *mischaracterization of the population (or even the sample). Furthermore, they suggest the need to approach such analyses*
192 *qualitatively, especially when samples sizes are insufficient for capturing the true isotopic variation.*

193

194 2.2 In-situ sampling methods cannot mimic when and where plant roots access water

195 The distribution of roots in the subsurface is often the first-order constraint over potential water
196 sources: uptake is unlikely to occur from zones that lack functional roots (but see papers on hydraulic
197 redistribution and mycorrhizal-mediated water transport; e.g., Augé, 2001; Dawson, 1993). It should be noted,
198 however, that roots differ in their functional or physical roles, e.g., anchorage and transport in suberized root
199 tissues versus resource acquisition in un-suberized tissues (Taiz & Zeiger, 2010). Furthermore, the role of a
200 root also depends on the interplay between its water potential and the surrounding soil's water potential, the
201 latter of which is a function of soil texture, water content and osmotic potential. These factors vary across
202 species, time and space, resulting in uptake dynamics with subtleties that are difficult to match with the
203 sampling methods used by researchers.

204 Some *in-situ* approaches sample water across a membrane using a pressure gradient, mimicking the
205 process used by plant roots (Steudle, 1994). For instance, suction lysimeters use buried porous ceramic cups
206 with a suction force applied to extract freely draining water and pore water. Depending on the ceramics'

207 porosity and the applied suction force, these lysimeters can extract water held at matric tensions reaching
208 -10^3 hPa (Sprenger, Herbstritt, & Weiler, 2015). Another benefit is that they allow repeated sampling of
209 particular locations in the root zone, which can potentially be useful to monitor small-scale temporal isotopic
210 variations of subsurface water. Importantly, where suction lysimeters differ from plant roots is the timing of
211 water uptake and the sizes of sampled pores: much of the applied potential may be satisfied following rainfall
212 events, and larger more conductive pores in the ceramic material may disproportionately transmit water
213 (Hansen & Harris, 1975; Weihermüller et al., 2007). This contrasts with the natural behavior of plant roots
214 when there is generally low transpirational demand after rainfall events (because atmospheric humidity is
215 high) and when soil water bypasses the rooting zone as it drains rapidly downward through the largest pores
216 (Brooks, Barnard, Coulombe, & McDonnell, 2010). Consequently, the isotopic composition of the water
217 extracted by suction lysimeters may differ from the surrounding pore waters that are later used by plants;
218 details of this process, however, have not been quantified experimentally.

219 Gas-permeable membranes have recently been used to extract subsurface water vapor *in-situ* for
220 direct isotope analysis to characterize the isotopic composition of the unsaturated zone (Oerter, Perelet,
221 Pardyjak, & Bowen, 2017; Rothfuss, Vereecken, & Brüggemann, 2013; Volkmann & Weiler, 2014; West,
222 Patrickson, & Ehleringer, 2006). This approach allows for characterizing water vapor that has equilibrated with
223 the liquid water in soils, reflecting water across a large variety of pores that surround the membrane probe.
224 For translating vapor isotope measurements into liquid water isotope data (relative to Vienna Standard Mean
225 Ocean Water, V-SMOW), calibration standards need to be generated by combining dry substrate from the
226 study site with waters of known isotopic composition. With this, potentially-confounding effects of isotopic
227 fractionation from clays or organic matter (Chen, Auerswald, & Schnyder, 2016; Oerter et al., 2014) can be
228 implicitly included in the calibration. To correct for fractionation effects during equilibrium exchange between
229 liquid water and vapor (Majoube, 1971), standards must be measured across a temperature range similar to
230 that of each sampling depth (Rothfuss et al., 2013). Limitations in the practical application of the *in-situ*
231 equilibration method can arise from condensation, mixing and diffusion of water vapor in the tubes leading
232 from the membrane to the laser spectrometer.

233 Although these and other *in-situ* sampling methods have been developed to characterize isotope
234 ratios in root-zone water (Orlowski, Pratt, & McDonnell, 2016; Sprenger et al., 2015), these methods may

235 sample differently across the subsurface spatiotemporal heterogeneities that we know to occur. It also needs
236 to be recognized that plants may root across each soil horizon (Dekker & Ritsema, 1996; Stewart, Moran, &
237 Wood, 1999), which may further complicate practical attempts to isotopically characterize plant water
238 sources. In conclusion, we advocate for choosing the sampling technique(s) depending on the subsurface
239 properties of the study site and we provide some practical recommendations in Sect. 5.

240

241 2.3 *In-lab extraction methods homogenize water across functionally distinct pores*

242 For applications where *in-situ* sampling is not ideal, root-zone water can also be extracted – as vapor
243 or liquid – in the laboratory from samples collected in the field. Being relatively cheap and technically easy,
244 augering into soils is common practice for collecting samples and characterizing subsurface properties up to a
245 few meters depth. However, augering is destructive, and thus repeated augering in a small area could
246 fundamentally alter the local infiltration pathways. Moreover, augering only provides integrated samples at
247 any particular depth, and thus the mixture of waters with distinct isotope ratios in any chunk of soil cannot be
248 resolved and the spatiotemporal resolution of such isotope data sets remains generally low (Landon, Delin,
249 Komor, & Regan, 1999).

250 Various techniques are used for extracting water from porous media in the laboratory, most
251 commonly cryogenic vacuum distillation and direct water vapor equilibration, that have been extensively
252 tested and evaluated in multiple studies (e.g., Araguás-Araguás, Rozanski, Gonfiantini, & Louvat, 1995; Kübert
253 et al., 2020; Orłowski et al., 2016; Sprenger et al., 2015; Thoma, Frentress, Tagliavini, & Scandellari, 2018;
254 Volkmann, Kühnhammer, Herbstritt, Gessler, & Weiler, 2016). Analytical uncertainties of cryogenic vacuum
255 distillation can result from incomplete extraction leading to partial distillation from the sample such that the
256 extracted water is isotopically lighter than that in the original sample (Stoll, Hissler, & Legout, 2014;
257 Thielemann, Gerjets, & Dyckmans, 2019). While cross-laboratory comparisons have attempted to quantify
258 errors involved in cryogenic extraction, such tests usually use soil samples that have been oven-dried at very
259 high temperatures (e.g., 105°C) and then spiked with water of known isotopic composition (e.g., Orłowski et
260 al., 2018). This approach might, however, introduce variable isotopic fractionation processes associated with
261 the re-hydration of oven-dried matrix materials, which is not representative of real-world soils (Gaj, Kaufhold,
262 & McDonnell, 2017; Sprenger et al., 2015). This suggests that such tests of the cryogenic extraction method

263 might exaggerate the true isotopic uncertainties (Newberry, Nelson, & Kahmen, 2017). Furthermore, because
264 of the extremely low water potentials used in cryogenic distillation, small residual water pools (e.g.,
265 hygroscopic and biologically-bound water) are collected that would rarely, if ever, be usable by plants. Thus,
266 particularly for soil samples with low moisture and/or high clay contents, cryogenic extraction may not
267 perfectly retrieve soil-water isotope ratios that match the water that is specifically available to plants.
268 Although cryogenic extraction is widely used, systems and user protocols can vary across laboratories
269 (Orlowski et al., 2018) and thus, uncertainties introduced by this method should always be quantified.

270 The direct equilibration method allows for water vapor of a moist soil sample to be measured directly
271 with a laser spectrometer in the lab (Hendry, Schmeling, Wassenaar, Barbour, & Pratt, 2015; Mattei et al.,
272 2019; Wassenaar, Hendry, Chostner, & Lis, 2008). Here, water vapor is extracted from a substrate sample in a
273 tightly sealed bag or container, taking advantage of the equilibrium vapor-liquid isotopic offset (Majoube,
274 1971). Similar to the in-situ equilibration method, vapor measurements are calibrated relative to V-SMOW by
275 measuring alternately water vapor from bags filled with isotope standards (Garvelmann, Kulls, & Weiler,
276 2012; Wassenaar et al., 2008). Uncertainties in the measured isotope ratios can increase when the water
277 content in the bag becomes too small (e.g., <3g in a 1-L bag; Hendry et al., 2015); also, volatile organics in the
278 water vapor, either already in the sample or due to microbial activity, can cause analytical interferences
279 (Gralher, Herbstritt, Weiler, Wassenaar, & Stumpp, 2018; Hsieh, Savin, Kelly, & Chadwick, 1998).

280 Other, less frequently used water extraction methods, such as centrifugation, mechanical squeezing,
281 azeotropic distillation and microwave distillation have also been evaluated for their capability to retrieve
282 representative root-zone water samples for isotope analysis (e.g., Adams et al., 2020; Figuéroa-Johnson,
283 Tindall, & Friedel, 2007; Kelln, Wassenaar, & Hendry, 2001; Munksgaard, Cheesman, Wurster, Cernusak, &
284 Bird, 2014). Some limitations of the methods have been identified, e.g. that squeezing and centrifugation yield
285 comparable results to direct equilibration only when used for coarse soils with >20% water content (Orlowski
286 et al., 2016); however, more testing is needed across a large range of soil types and extraction conditions to
287 facilitate a more widespread application of these alternative methods.

288 Whereas in-lab extraction methods typically cannot deliver the high-frequency measurements useful
289 for matching xylem water to the constantly changing isotope values in the root zone – e.g., because frequent
290 coring may fundamentally alter the infiltration properties of the subsurface – *in-situ* methods rarely provide

291 insights into the spatial variability in $\delta_{root-zone}$. Choosing one sampling or extraction strategy over others is
292 implicitly a compromise that requires considerations of its inherent limitations and the associated
293 uncertainties (but see Sect. 4 and 5).

294

295 **3. Xylem water: Characterizing and sampling across natural variations in isotope** 296 **mixtures**

297 Our review of the spatiotemporal heterogeneity within root-zone water endmembers points towards
298 the importance of their careful characterization; similarly, variability in xylem water isotopic composition
299 (δ_{xylem}) should be carefully considered when defining xylem water as a mixture of root-zone water sources.

300 An inability to attribute xylem water mixtures to potential root-zone water endmembers may not only be due
301 to under-sampling of subsurface water sources, but also due to challenges in determining δ_{xylem} values.

302 While it has been previously assumed that the isotope ratios of water extracted from plant xylem exactly
303 reflect that of the water taken up by roots, we discuss evidence suggesting that δ_{xylem} should be used as an
304 approximation – not an exact reflection – because fractionation effects and heterogeneity create
305 uncertainties.

306

307 *3.1 Uncertainties in xylem water isotope values associated with extraction, analysis and natural* 308 *variability within the plant*

309 Flow through vascular plants involves many of the same processes as flow through soils, such as
310 preferential flow through certain pores and mixing of new inputs with stored water. Thus, heterogeneities in
311 the xylem water properties arise and the best practices for sampling plants may be very similar to those for
312 soils: sample extensively and often. However, paralleling the measurement challenges in plants with those in
313 soils, plant-sampling methods are limited in their ability to extract the water flowing in xylem (without also
314 extracting stored waters) and are not weighted by the relative importance to the transpiration stream. Most
315 commonly, tree tissue is obtained from coring tree trunks or sampling thicker branches with bark (Dawson &
316 Ehleringer, 1993), from which xylem water is extracted via cryogenic vacuum distillation, microwave extraction
317 or high-pressure mechanical squeezing (e.g., Koeniger, Marshall, Link, & Mulch, 2011; Millar, Pratt, Schneider,

318 & McDonnell, 2018). Alternative methods include xylem-water vapor sampling with the direct equilibration
 319 method (Millar et al., 2018), or with vapor-permeable membranes implanted into the tree's water-conducting
 320 xylem (Marshall, Cuntz, Beyer, Dubbert, & Kuehnhammer, 2020; Volkman et al., 2016). Analytical
 321 uncertainties associated with each of these extraction methods and subsequent isotope analysis can be
 322 substantial (but see reviews of Martín-Gómez et al., 2015; Millar et al., 2018; West, Goldsmith, Matimati, &
 323 Dawson, 2011), and thus requires a careful selection of an approach based on the research's specific goal, as
 324 well as on the sample types, costs and needed precision.

325 In addition to analytical errors, uncertainties in δ_{xylem} can arise due to the natural spatiotemporal
 326 heterogeneity of water flow in plants. E.g., different parts of the tree crown can be supplied by different flow
 327 pathways through the tree, potentially connected to roots tapped into different water pools (Schulte &
 328 Brooks, 2003; M. H. Zimmermann, 1983). A single snapshot of 57 trees across 1ha (Goldsmith et al., 2019)
 329 showed strong variation in δ_{xylem} among individual branches (intra-crown variability; Table 2), which was
 330 attributed to sectorality. Xylem sectorality is also hypothesized to explain the 6.5-9.3‰ variation in $\delta^{18}O$
 331 measured in single redwood trees that were 80-107m tall (T. E. Dawson, personal communication, May 2020).
 332 Additionally, it is assumed that heartwood water does not substantially contribute to transpiration, but it is not
 333 well known how hydraulically isolated heartwood water remains until it is needed, e.g., during drought (Scholz,
 334 Philips, Bucci, Meinzer, & Goldstein, 2011). Systematic comparisons of sapwood versus heartwood are needed
 335 to better inform best practices for stem sampling (but see White et al., 1985). While the generality of these
 336 findings for all plant types and environments is yet unknown, they suggest that single xylem water samples can
 337 only provide a partial view into any tree's water sources.

338 Post-uptake processes in the plants themselves can also affect the isotope composition of xylem
 339 water. For herbaceous plants, transpiration occurs in most above-ground tissues, such that evaporative
 340 fractionation at the leaf-atmosphere interface leads to isotopic enrichment compared to source water (Craig &
 341 Gordon, 1965; Farquhar et al., 1993; Helliker & Ehleringer, 2000). In this case, herbaceous-plant tissue should
 342 be sampled from root crowns as they are the least isotopically variable and seem most reliable (Barnard, Bello,
 343 Gilgen, & Buchmann, 2006). For trees, evaporative fractionation should also be considered for sections close
 344 to the leaf-atmosphere interface, or in green stems that contain stomata or lenticels where isotopically
 345 enriched water may diffuse backwards and re-mix (Ehleringer, Roden, & Dawson, 2000; Lehmann et al., 2018).

346 As a result, δ_{xylem} values within small stems or leaves can differ substantially from each other and from those
347 within the larger trunk.

348 There can also be meaningful variations in δ_{xylem} values among closely spaced individuals, that
349 should not be ignored through averaging. While often site-level or species-level plant-source water inferences
350 are sought, pursuing those should include accounting for among-tree (or even within-tree) variations in water
351 sources (Figure 3). Combining sample data from across parts of the plant to capture this multi-scale
352 hierarchical heterogeneity (e.g., twigs, branches or trunk cores of trees) should complement xylem water
353 studies, to enable reporting uncertainties in individual δ_{xylem} values.

354

355 3.2 Water-transport lags within trees complicate endmember mixing analyses

356 Time lags from water traveling from roots to stem to twig exist, so we should understand the range of
357 temporal variations in root-zone water over recent times and not just at the instant of sampling. Soil water
358 and xylem water are often collected at the same time, and thus endmember-mixing analyses implicitly neglect
359 the lags between time of uptake and time of water reaching the stem or twig that is sampled. Those travel
360 times and transport velocities of water in the plants are not well quantified. Some studies have combined
361 measurements of stable water isotopes and sap flux to estimate the time from uptake to transpiration, mostly
362 showing that peak tracer concentrations lag inputs by one day to one week (e.g., Gaines, Meinzer, Duffy,
363 Thomas, & Eissenstat, 2016; Schwendenmann, Dierick, Kohler, & Holscher, 2010), but also lag times as long as
364 2-3 weeks have been observed in 1-m diameter 50-m tall trees (Meinzer et al., 2006). As a consequence of the
365 wide range of water residence times that can exist in trees, synchronizing the sampling of subsurface material
366 with the sampling of plant material for subsequent isotope analysis remains challenging.

367

368 3.3 Fractionation during root water uptake should not be ignored

369 The assumption that the isotope composition of root-zone water exactly matches that of the plant's
370 xylem water is based on early investigations that found no evidence for isotope fractionation during the
371 uptake process (Washburn & Smith, 1934). Several subsequent studies also reported data that supported this
372 claim (Dawson & Ehleringer, 1991; Walker & Richardson, 1991; U. Zimmermann et al., 1967). In the first paper

373 we could find on this topic, Washburn and Smith (1934) grew plants hydroponically and measured the change
374 in density of the remaining water as a proxy for isotopic fractionation; no change in density was seen after
375 approximately 99% of the hydroponic water was removed by transpiration. These results formed the initial
376 basis for the assumption that plants do not discriminate against any isotopologues of water during uptake.
377 However, many of the early investigations used only one isotope (either ^2H or ^{18}O) because their analysis was
378 not easy, reliable or routine as it has become today. This, in addition to smaller sample sizes, may be a reason
379 why any isotope fractionation effects, if they were present, may have not been detected. Using both $\delta^2\text{H}$ and
380 $\delta^{18}\text{O}$, with which the deuterium excess can be calculated, makes it far easier to identify whether isotopic
381 fractionation has altered a water sample (Dawson & Simonin, 2011; Sprenger et al., 2016).

382 Furthermore, the studied plants in Washburn and Smith (1934) were never water limited; however, in
383 unsaturated conditions where strong potentials are at play in the root-substrate interface, one may observe
384 isotopic fractionation upon root water uptake. Evidence suggests that (hydrogen) isotopic fractionation during
385 root water uptake can occur in some specialized groups of plants that can live in salt water or saline soils
386 (Ellsworth & Williams, 2007; G. Lin & Sternberg, 1993). Recent potted plant experiments found that isotope
387 values in xylem water were consistently lower than those in root-zone water (e.g., with absolute offsets in $\delta^2\text{H}$
388 averaging 9.2‰ in Vargas et al. (2017), 10.6‰ in Barbeta et al. (2020), and ranging from 2.9 to 15.6‰ in Poca
389 et al. (2019)). The processes leading to these isotopic offsets are not well understood, although the existing
390 studies propose some possible explanations. While uptake in most plants is an advective mass-flow process
391 (apoplastic flow), Poca et al. (2019) speculated that uptake can also involve transmembrane water transport
392 through aquaporins, which may discriminate against heavy isotopes. In contrast, Barbeta et al. (2020)
393 hypothesized that xylem water is isotopically lighter than root-zone water because xylem-water samples
394 contain isotopically depleted water stored in stem tissue. It is also possible that isotopic fractionation occurs
395 during liquid-vapor phase transitions in the root-zone pore spaces located in close proximity to the plant roots.
396 As first outlined in Allison, Barnes, Hughes, and Leaney (1984) and later elaborated on by others including
397 Vargas et al. (2017), temperature and flow dynamics in the subsurface can yield liquid-vapor exchanges that
398 are not explained by equilibrium fractionation factors and have directional effects on subsurface isotope
399 values.

400 These recent studies suggest that for some plant species and environmental conditions, root water
401 uptake may cause xylem water to be isotopically depleted relative to its source water, whereas other studies
402 indicate that source and xylem waters are seemingly mismatched because of isotope variations within the
403 sampled materials. Experiments to better understand this depletion may need to focus on small-scale
404 variations in water inside roots, xylem vessels or tracheids, and other storages. Otherwise, until the physical
405 and chemical processes underlying these apparent fractionations are understood well enough to account for
406 them mechanistically, errors should be assumed to avoid compromising the validity of endmember mixing-
407 model analyses.

408

409 **4. Uncertainties abound: Determining mixture and endmember isotopic**
410 **signatures is technically challenging and associated uncertainties are often unknown**

411 While the question often arises “Which method is the right one?” for identifying the root-zone water
412 endmembers that plants may access, we should recognize that our tools are unlikely to be as flexible and exact
413 as any plant root is. Furthermore, every step towards quantifying an isotope ratio introduces uncertainty. This
414 poses real technical limitations for applying endmember measurements, rendering them only as an
415 approximation and demanding the assumption that δ_{xylem} and $\delta_{root-zone}$ values are uncertain. These
416 uncertainties – associated with natural variability, sampling limitations, and measurement or extraction
417 analytical errors – vary widely, depending on the system conditions and how water is sampled, prepared,
418 extracted, and analyzed.

419 Only few studies have specifically investigated the δ_{xylem} and $\delta_{root-zone}$ uncertainties that can
420 naturally occur due to the heterogeneity inherent in natural systems, and some examples are shown in Table
421 1. These values can be large for both δ_{xylem} and $\delta_{root-zone}$, suggesting that many samples need to be
422 collected and analyzed to quantify the natural uncertainties in the mixture and endmember isotopic
423 signatures, which then allows for more robust endmember-mixing analyses (e.g., such as in Figure 3c).

424

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425 *Table 1: Ranges of natural isotopic variability, expressed as 1 standard deviation (σ) or mean isotopic difference (Δ), that*
 426 *can occur within trees and soils. These values only provide a limited selection of isotope uncertainties and more detailed*
 427 *analyses have been carried out elsewhere (references in Sect. 2.1 and 3.3).*

Source of variability	Experimental details	Observed natural variability (‰ VSMOW)		Reference
		$\delta^{18}\text{O}$	$\delta^2\text{H}$	
a Within the tree crown	<i>P. abies</i> branch xylem water (σ of 5 samples, averaged across 3 trees)	1.6	4.4	Goldsmith et al. (2019)
b Among-tree variability within plot	<i>P. abies</i> branch xylem water (σ of 4-8 trees per plot, averaged across 71 plots)	0.8	2.1	Allen, Kirchner, et al. (2019)
c Laterally in deep soil	Soil water from 40-50cm depth across 1ha (σ , n=8)	1.0	7.1	Goldsmith et al. (2019)
d Laterally in shallow soil	Soil water from 0-10cm depth across 1ha (σ , n=150)	1.7	10.6	Goldsmith et al. (2019)
e Isotopic separation during root water uptake	Irrigated sealed pots with <i>Persea Americana</i> , $\Delta = \delta_{\text{soil}} - \delta_{\text{xylem}}$ (mean Δ , n=32)	1.1	9.2	Vargas et al. (2017)

428

429 Table 2 shows some typical uncertainty values due to sample extraction and analysis, which were
 430 retrieved from studies specifically targeted to quantify these uncertainties. However, most of the studies that
 431 partially tested and evaluated laboratory and field-based extraction methods for soils (Orlowski et al., 2016;
 432 Orlowski et al., 2018; Sprenger et al., 2015; Sprenger et al., 2018; Thoma et al., 2018) and plants (Newberry et
 433 al., 2017; Martín-Gómez et al., 2015; Millar et al., 2018) have targeted the discussion on uncertainties at
 434 certain problems and thus are not directly transferable to other laboratory infrastructures or sample media. In
 435 fact, such tests can be ambiguous; for example, studies that have attempted to quantify the difference
 436 between δ_{xylem} and $\delta_{\text{root-zone}}$ face practical limitations such as when soils also undergo evaporation which
 437 likely causes isotopic enrichment of soil water and confounds inferring analytical errors (Millar et al., 2018;
 438 Newberry et al., 2017). The uncertainties in Table 2 are further compounded by the fact that the soil water
 439 measurements are not necessarily measurements of the specific soil-water that a plant might extract from the
 440 root zone; however, this additional uncertainty is not well quantified (Sect. 2). Therefore, the uncertainties in
 441 Table 2 should rather be used to guide decisions about sampling- and analysis procedures.

442

443

444

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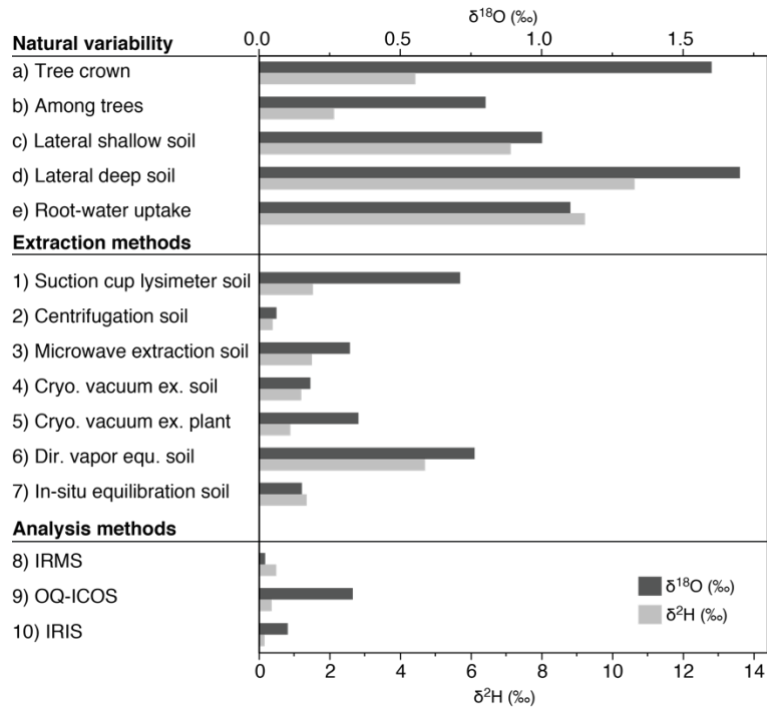
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446 *Table 2: Analytical uncertainties of commonly-used extraction and measurement methods for stable water isotopes in soil*
 447 *and plant samples. Error was quantified as the mean absolute deviation from an isotope reference value (mostly that of*
 448 *spike water) and repeatability was quantified as one standard deviation of that mean. These values only provide a limited*
 449 *selection of isotope uncertainties and more detailed method comparisons have been carried out elsewhere (references in*
 450 *Sect. 2.2, 2.3 and 3.1).*

Extraction methods	Experimental details	Metric	$\delta^{18}\text{O}$ (‰ VSMOW)	$\delta^2\text{H}$ (‰ VSMOW)	Reference	
1	Suction lysimeter (70–75kPa), IRMS	Soil water, spiked sandy loam (n=10)	Error Repeatability	0.68 0.71	1.9 1.5	Thoma et al. (2018)
2	Centrifugation (5000rpm, 15min), OA-ICOS	Soil water, spiked silty sand, 20% GWC (n=5)	Error Repeatability	0.19 0.06	1.08 0.36	Orlowski et al. (2016)
3	Microwave extraction (330W, 15min), OA-ICOS	Soil water, spiked silty sand, 20% GWC (n=5)	Error Repeatability	0.57 0.32	24.95 1.47	Orlowski et al. (2016)
4	Cryogenic vacuum distillation (98°C, 45min), OA-ICOS	Soil water, spiked silty sand, 20% GWC (n=5)	Error Repeatability	0.71 0.18	5.54 1.17	Orlowski et al. (2016)
5	Cryogenic vacuum distillation (100°C, 210min), IRMS	Xylem water, root crown, irrigated open pots with <i>Triticum aestivum</i> L., (n=5)	Error Repeatability	Not reported 0.35	0.86	Millar et al. (2018)
-	Cryogenic vacuum distillation (90°C, 120min), IRMS	Xylem water, irrigated sealed pots with <i>Salix viminalis</i> (n=68)	Error Repeatability	0.84 1.13	Not signif. Not reported	Newberry et al. (2017)
6	Direct vapor equilibration method with bags (6d), OA-ICOS	Soil water, spiked coarse sand, medium sand, coarse silt, 8-50% GWC (n=9)	Error Repeatability	0.52 0.76	2.87 4.67	Mattei et al. (2019)
7	<i>In-situ</i> equilibration method with membranes (DDS, TI), IRIS	Soil water, slightly clayey silt (n=9)	Error Repeatability	0.12 0.15	1.10 1.32	Volkman and Weiler (2014)
Analysis methods						
8	IRMS (Thermo Fischer Delta Plus Advantage mass spectrometer (Thermo Fisher Scientific Inc., Massachusetts, USA) connected to a GFL 1086 equilibration device)	Water, 10 replicates (n=13)	Repeatability	0.02	0.46	Penna et al. (2012)
9	OA-ICOS (Los Gatos Research Inc., off-axis integrated cavity output spectroscopy model DLT-100 version 908-0008 or newer)	Water, last 8 of 18 injections (n=72)	Repeatability	0.33	0.33	Penna et al. (2012)
10	IRIS (Picarro Inc., model L1102- <i>i</i> liquid analyzer or newer)	Water, last 8 of 18 injections (n=72)	Repeatability	0.1	0.13	Penna et al. (2012)

451 Figure 4 visually contrasts the values from Table 1 and Table 2 and demonstrates that the ranges of
 452 isotope variations attributed to natural heterogeneity mostly exceed analytical errors that have been the
 453 primary focus of past critiques on isotope-based plant-water uptake studies. Thus, potentially large
 454 uncertainties in δ_{xylem} and $\delta_{root-zone}$ values do not imply that stable water isotopes cannot be used to study
 455 plant water sources. Figure 4 rather suggests that errors and uncertainties must be recognized and
 456 incorporated into mixing-model analyses. Even if these errors and uncertainties cannot be quantified in a
 457 given study, assuming values with magnitudes similar to those in Table 1 and Table 2 would be a reasonable
 458 alternative.

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459

460 *Figure 4: Natural variability of isotope values in soils and plants, and uncertainties of isotope values (repeatability) due to*
 461 *extraction and analysis methods. Information about the data and references are provided in Table 1 and Table 2.*

462

463 5. Looking forward: designing useful experiments given endmember challenges

464 Isotope-based endmember mixing models are widely used to quantitatively determine which root-
 465 zone water sources are taken up by plants. Given that both the mixture and endmember terms can be
 466 challenging to characterize, we should assume mixing-model solutions to be inexact and design studies that
 467 maximize the isotopic contrast between endmembers and/or minimize potential uncertainties. As a first step,
 468 uncertainties should be estimated or assumed to theoretically determine what the smallest isotopic difference
 469 between two endmembers needs to be to enable a robust endmember-mixing analysis (see Sect. 4, and
 470 example in Rothfuss & Javaux, 2017).

471 To summarize and conclude, we provide some specific recommendations to maximize signal-to-noise ratios
 472 and thus to enable more confidently inferring plant source waters in physiological, ecological or eco-hydrologic
 473 studies. These approaches (and any sampling design) should always be adapted to the specific properties of
 474 the study site: the soil and/or substrate type and structure, the climate, and the hydrological setting (e.g.,
 475 hypothesized range of water sources), and of course, any knowledge of rooting patterns.

476 1) Design experiments to maximize the isotopic contrast between endmembers

477 Exploit extreme isotopic anomalies in plant water sources

478 Stable water isotopes may be best suited for identifying potential plant water sources such as fog,
479 mist or dew (Dawson, 1998; Hill, Dawson, Shelef, & Rachmilevitch, 2015), or rock moisture in the
480 deep weathered bedrock underneath hillslope soils (Oshun, Dietrich, Dawson, & Fung, 2016;
481 Schwinning, 2010), where values deviate strongly from soil water. Other isotopic contrasts between
482 subsurface waters may occur due to mineral-water interactions that lead to strongly fractionated
483 pore water (Y. Lin & Horita, 2016; Oerter et al., 2014), or when groundwater is more depleted than soil
484 water because groundwater recharge was fed mostly by isotopically-light snowmelt (Dawson &
485 Ehleringer, 1991). We can design sampling campaigns specifically to target these isotopic anomalies,
486 or target circumstances where the isotopic differences between the endmembers are large, so that
487 we can more robustly use mixing models.

488 Ask questions about root-zone water uptake during the driest conditions

489 During dry conditions, root-zone water isotope profiles will be more monotonic (mainly due to
490 evaporative fractionation near the soil surface), compared to after-precipitation conditions when
491 infiltrating water mixes with pre-event soil water (Sect. 2). Thus, distinguishing among shallow and
492 deep root-zone water sources with an endmember-mixing model is inherently easier (i.e., less
493 uncertain) during dry periods and in dry regions.

494 Study water uptake after precipitation events that follow dry periods

495 Rather than orienting research questions around depth of water uptake, similarly useful insights can
496 be gained by asking whether plants are using recent precipitation (or snowmelt) event water. This
497 approach requires that the isotope signal of the event water is very distinct from that already stored
498 in the subsurface (i.e., pre-event water). Thus, sampling root-zone and xylem water before and after
499 events enables us to see whether recent water is taken up by the plant roots (Oerter and Bowen
500 (2017), Zhang, Jiang, Wang, Jiao, and Wang (2018)).

501 Perform artificial labelling experiments

502 Applying isotopically enriched or depleted water to the root zone can increase the isotopic differences
503 between the plant source water endmembers in the mixing analyses. Labelled water can be sprinkled
504 on experimental plots to better discern the contribution of “irrigation” versus “pre-irrigation” water

505 sources to plant water (Grossiord et al., 2014). Alternatively, labelled water can be used to mark
506 specific locations of the root zone to investigate the distribution of active roots (Beyer et al., 2016) or
507 hydraulic redistribution processes (Zapater et al., 2011).

508 **2) Quantify and minimize uncertainties in endmembers and mixture**

509 Quantify xylem water isotopic heterogeneity at the plant- or plot-level

510 While isotopic heterogeneity should be expected within soil samples and among soil samples at the
511 same depths (Sect. 2), it can also be relevant among xylem samples within and among plants (Sect. 3).
512 While identifying these heterogeneities may be of interest for some specific research questions,
513 usually we are more interested in species-level or plot-level inferences, and thus want to include
514 within-tree variability as an uncertainty term. Optimally, not only individuals are sampled, but also
515 multiple twigs from individuals, so that all δ_{xylem} values can then be incorporated in any endmember-
516 mixing analysis, e.g. by using iterations of mixing models for all permutations of individual sample
517 values (Figure 3).

518 Use the dual-isotope approach

519 Both $\delta^{18}\text{O}$ and $\delta^2\text{H}$ can now be analyzed simultaneously at very low costs, so that isotopic
520 fractionation effects can be tracked and quantified as deviations from precipitation using deuterium
521 excess or line-conditioned excess (Landwehr & Coplen, 2006). If the relationship between $\delta^{18}\text{O}$ and
522 $\delta^2\text{H}$ varies with depth across the root-zone profile (e.g., Figure 2a), both isotopologues can be used as
523 individual endmembers in mixing models. Alternatively, the deuterium excess or line-conditioned
524 excess values themselves can be used to characterize depth-dependent endmembers and the
525 mixture.

526 Conduct potted-plant experiments

527 Potted-plant experiments allow for controlling and monitoring all input and output water fluxes,
528 which is useful for tracking variations in δ_{xylem} and $\delta_{root-zone}$ at higher precision and resolution than
529 would be possible in most natural systems. Because the complexity inherent in most natural systems
530 is reduced in such experiments, we can study individual processes in greater detail (such as
531 fractionation effects during plant-water uptake; Sect. 3.3). Although potted-plant experiments may

532 never fully represent natural systems, a well-designed set-up allows for informative comparison
 533 analyses between different environmental conditions and plant species (Kawaletz et al., 2014).

534 Monitor isotopic variations in root-zone and xylem water at high temporal resolution

535 New sampling techniques, such as the *in-situ* equilibration method with membrane probes,
 536 potentially allow for measuring δ_{xylem} and $\delta_{root-zone}$ at sub-hourly resolution over periods of weeks
 537 and months (Sect. 2.2). This might be particularly practical for detecting temporal isotopic anomalies
 538 in the root zone (due to fractionation or mixing of new water inputs), and thus may allow for more
 539 robust source water attribution. Isotope time series data collected with such methods may also be
 540 well suited for calibrating mechanistic models (Knighton et al., 2020).

541

542 **6. Summary and conclusions**

543 Stable isotopes of water can provide powerful insights into plant water sources, however, accurately
 544 determining from when and from where plants take up water requires us to account for the potential sources
 545 of uncertainties and limitations associated with the isotope approach. Isotope-based endmember mixing
 546 models should only be used to distinguish among highly distinct and well-characterized plant water sources.
 547 This means that the differences between endmembers need to be much larger than the uncertainties
 548 associated with sample extraction, analysis or modeling in order to yield robust and unambiguous results
 549 (Figure 4). Nonetheless, numerous studies have shown truly distinct endmembers, enabling robust inferences
 550 on plant water sources, and advancing our understanding of plant water uptake (see references in Sect. 1).

551 Ideally, all endmembers of a mixture should be known. However, sampling all endmembers is often
 552 not practicable due to high sample extraction costs, technical limitations, or unpredictable root distributions.
 553 In those cases when not all endmembers can be quantified, we know that uncertainties still exist and thus
 554 their consequences for the endmember-mixing model results should be acknowledged. Although we define
 555 distinct endmembers, and thus drastically simplify and discretize the complex subsurface water flow
 556 processes, endmember-mixing models still provide a route towards new understanding that is not always
 557 compromised by recent findings about isotopic variations in the root zone and in the plant. Suggestions for
 558 best using this this route are provided in Sect. 5.

559 In addition to the uncertainties associated with the endmembers of plant water sources, we need to
560 acknowledge the limitations of the concept behind isotope-based endmember mixing analyses. While
561 technology improves, it remains unlikely that mixing-model analysis will transition from a robust comparative
562 method to one that provides exact information on depths of root water uptake. In other words, isotope-based
563 endmember mixing models allow us to identify which water sources the plant “uses” but it cannot always help
564 us to identify the sources it “depends on”. Nonetheless, new technologies have significantly increased
565 temporal and spatial sampling frequencies, which can mitigate and constrain the uncertainties discussed in
566 this paper. We are hopeful that continuous progress and method development will provide new insights on
567 plant- and ecosystem-level water relations.

568

569 **Authors’ contributions**

570 JF, SA, CG and TD conceived the ideas and designed methodology, hence collected and reviewed the
571 references and data. JF and SA led the writing of the manuscript with substantial inputs from CG and TD. All
572 authors contributed critically to the drafts and gave final approval for publication.

573

574 **Data accessibility**

575 Data for this article consist of lists of natural variations and analytical uncertainties in stable water isotope
576 values that were pulled from 10 published studies and that are presented in Table 1 and Table 2.

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