Received: 2 December 2021 | Revised: 1 February 2022 | Accepted: 10 March 2022

DOI: 10.1002/eco.2417

RESEARCH ARTICLE

WILEY

# Phloem water isotopically different to xylem water: Potential causes and implications for ecohydrological tracing

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**Funding information**
NSERC Discovery Grant; NSERC CREATE in Water Security Grant; SNSF Ambizione Grant, Grant/Award Number: 179978; American Geophysical—Horton Research Grant 2019

## Abstract

The stable isotopes of hydrogen and oxygen in xylem water are often used to investigate tree water sources. But this traditional approach does not acknowledge the contribution of water stored in the phloem to transpiration and how this may affect xylem water and source water interpretations. Additionally, there is a prevailing assumption that there is no isotope fractionation during tree water transport. Here, we systematically sampled xylem and phloem water at daily and subdaily resolutions in a large lysimeter planted with *Salix viminalis*. Stem diurnal change in phloem water storage and transpiration rates were also measured. Our results show that phloem water is significantly less enriched in heavy isotopes than xylem water. At subdaily resolution, we observed a larger isotopic difference between xylem and phloem during phloem water refilling and under periods of tree water deficit. These findings contrast with the expectation of heavy-isotope enriched water in phloem due to downward transport of enriched leaf water isotopic signatures. Because of previous evidence of aquaporin mediated phloem and xylem water transport and higher osmotic permeability of lighter hydrogen isotopologues across aquaporins, we propose that radial water transport across the xylem–phloem boundary may drive the relative depletion of heavy isotopes in phloem and their relative enrichment in xylem.

**KEYWORDS**
phloem water, stable isotopes, stem water storage, tree water relations, tree water source, tree water status, xylem water

## 1 | INTRODUCTION

Isotope tracing studies of plant water uptake often conceptualize tree water transport as a unidirectional process. Sampled xylem water is assumed to represent and integrate the broad suite of subsurface soil water and groundwater source reservoirs (Dawson & Ehleringer, 1991; Hervé-Fernández et al., 2016; Moreno-Gutiérrez et al., 2012; Volkmann, Haberer, et al., 2016). But this traditional

Significance Statement: Traditional approaches to investigating tree water sources use stable isotopes of water. This investigation is done by sampling xylem and using its signature to match available sources (e.g., soil water and groundwater). But this approach does not acknowledge the contribution of water stored within the tree to the xylem. The daily contribution of water in the phloem, a main water storage within the tree, could affect xylem water signature. We therefore investigated both xylem and phloem water signatures to understand how and when phloem water differs from xylem water. The difference between these pools has not been yet systematically explored and is critical as they affect tree water source interpretations.

  

approach does not acknowledge how integrated plant water transport occurs. Water is transported and stored in the phloem and can actively participate in the transpiration stream (Steppe et al., 2006). The contribution of phloem water to transpiration stream may affect xylem water isotopic composition and xylem water source interpretations.

Perhaps the reason that phloem and phloem water sampling has been ignored in isotope tracer studies of tree water use is linked to early explanations of tree water transport. Phloem and xylem water transport were presented as separated systems with distinct functions: phloem that transports photosynthates from leaves to roots and xylem that transports water and nutrients from roots to leaves, with little information on their hydraulic interactions as discussed in Pfautsch, Hölttä, and Mencuccini (2015). Perhaps this assumption of negligible phloem impact also relates to the fact that earlier work suggested too that phloem water transport was small (e.g., Zweifel & Häsler, 2000). These views have been somewhat overturned with more recent work where we now understand phloem−xylem transport as an integrated process, in which there is a complex bidirectional flow of metabolic products and water (Pfautsch, Hölttä, & Mencuccini, 2015). Thus, quantifying and accounting for phloem water isotopic signatures may in fact be needed, given the growing evidence of phloem hydraulic importance on tree water transport (Mencuccini et al., 2013; Sevanto et al., 2011; Steppe et al., 2006) and the often unexplained offset between xylem and soil water isotope values (Barbeta et al., 2020; de la Casa et al., 2021; Tetzlaff et al., 2021).

Tree hydraulic models and field experiments have now shown the tight hydraulic linkage between xylem and phloem. Water stored in phloem moves into xylem vessels sustaining tree water transport integrity and buffering rapid changes in xylem water potential (Pfautsch, Renard, et al., 2015; Sevanto et al., 2011; Steppe et al., 2006; Treydte et al., 2021). Thus, phloem−xylem water movement is analogous to flow in a porous medium, where the flow rate is proportional to the water potential gradient between xylem and phloem and modulated by the hydraulic conductivity across the xylem–phloem interface, influenced additionally by phloem osmotic pressure (Hölttä et al., 2006; Mencuccini et al., 2013). Despite phloem being smaller in volume than xylem, phloem has high elasticity (Kallarackal & Milburn, 1985) and can store large volumes of water and act as a capacitor to the transpiration stream (Pfautsch, Renard, et al., 2015). This bidirectional water transport between xylem and phloem occurs daily. The extent and magnitude of this process can be observed with tree stem measurements of diameter changes (De Schepper et al., 2012; Irvine & Grace, 1997; Lazzarin et al., 2019; Zweifel & Häsler, 2000). Dendrometers can capture these diel shrink–swell cycles in tree stems in high resolution (Drew & Downes, 2009), which reflects the hydration of inner bark and release of water into the xylem linked to transpiration driven negative pressure (Mencuccini et al., 2017; Zweifel et al., 2001; Zweifel & Häsler, 2000).

Notwithstanding the current knowledge on the general functioning of water transport between xylem and phloem, our understanding of subdaily variability in water exchange between xylem and phloem is still limited (Steppe et al., 2012; Treydte et al., 2021). Previous theoretical and empirical studies (Hölttä et al., 2009; Salomón et al., 2017; Sevanto et al., 2011; Steppe et al., 2012) indicate that phloem contribution to transpiration depends on variability in environmental conditions that drives the gradient of water potential between phloem and xylem, the volume of phloem and xylem tissue, the phloem capacitance and the hydraulic conductivity contrast between xylem and phloem.

Returning to our question of isotope tracing of plant source water, the evidence of radial water exchange between xylem and phloem and the daily contribution of phloem water storage to transpiration now appears to conflict with the assumption of no exchange. Because of evaporative enrichment of water at the leaf level during transpiration, and the descending transport of water and photosynthetic assimilates in phloem water, we would expect phloem water to be isotopically enriched in heavy isotopes relative to xylem water. The enrichment of phloem water relative to xylem water has been observed in some previous studies (Adar et al., 1995; Cernusak et al., 2005), but it is not consistent across the few existing observations on trees (Treydte et al., 2021; Wershaw et al., 1970). Notwithstanding our current understanding of xylem–phloem exchange, assessing the isotopic composition of phloem water throughout the diurnal cycle of xylem–phloem exchange is a key next step in this research area and potentially the way to better comprehend patterns of tree water use and the apparent fractionation of xylem water relative to its soil water pool. New insights into phloem water isotopic signatures, especially in high temporal frequency, could help improve mechanistic understanding of xylem water isotope ratios that cannot be explained by source water isotopic composition alone (Barbeta et al., 2020). Additionally, isotopic investigations of water flux across the phloem and xylem interface combined with stem diameter measurements could serve as aid to advance mechanistic understanding of phloem transport (Epron et al., 2019; Smith & Merchant, 2019).

Here, we present a 24-day investigation of the isotopic composition of phloem water and its relation to xylem water, over daily and subdaily changes in plant water status. We conduct high-frequency measurements of xylem and phloem isotopic composition, coupled with dendrometer measurements of stem diurnal change in phloem water storage, and transpiration rates using sap flow sensors in a large 2.5 $m^3$ lysimeter planted with *Salix viminalis*. An important aspect of this experimental design is the high transpiration rate, up to 12 mm/day (Benettin, Nehemy, Asadollahi, et al., 2021; Nehemy et al., 2021; Queloz et al., 2015) that allows observation of dynamic water transport. We leverage this dynamism to observe variability in xylem and phloem water isotopic values within the 24-day sampling period. We thus use this experimental design to ask two simple exploratory questions vis-à-vis isotope tracing and the composition of phloem/xylem water through time: (1) How do xylem and phloem isotope ratios differ? (2) How does daily use of phloem water and overall tree water status affect phloem−xylem isotope ratios?

## 2 | MATERIAL AND METHODS

We conducted our controlled experiment in a large lysimeter situated in an open field on the campus of École Polytechnique Fédérale de Lausanne (EPFL), in Switzerland (Figure 1) in 2018. The set-up consisted of a large fibreglass–polyester cylindrical tank, 2.5 m deep with a 1.12 $m^2$ surface area (Figure 1) (Benettin, Nehemy, Asadollahi, et al., 2021; Nehemy et al., 2021; Queloz et al., 2015). The lysimeter was filled with soil (50% loamy sand and 50% lacustrine sand from Lake Geneva) and planted with two willow cuttings (*S. viminalis*) originated from the same tree. Both soil and trees were placed and planted in 2012. The soil has remained undisturbed since then, and the trees regenerated after being cut down in 2014. The *S. viminalis* has been growing in these conditions from 4 years without disturbance prior to start of this experiment. The trees were about 3 m tall with a canopy cover of about 6 $m^2$ by the start of our experiment. The species were originally chosen because of *S. viminalis*'s ability to withstand both dry and wet conditions. The lysimeter sat on three load cells (HBM, Germany) connected to a digital transducer (AD103C, HBM). The weight of the lysimeter was recorded at 20 s intervals via a computer console (AD Panel32 software, HBM). Data from this experiment are available at Nehemy et al. (2020).

### 2.1 | Water inputs: Irrigation and precipitation

We irrigated the lysimeter to simulate different wetness conditions and changes in tree water status. We did this by monitoring soil water storage and irrigated as needed throughout the experiment. The irrigation events occurred at night to minimize changes in the isotopic signature of irrigation water due to evaporation. We irrigated the lysimeter with local tap water ($-12.01‰ \pm 0.08$ $\delta^{18}O$ and $-87.80‰ \pm 0.11$ $\delta^{2}H$) at the soil surface. The average irrigation was 30 mm per event (about three times the average daily transpiration), except for the three last events (22–24 June) that were each ~64 mm. We applied this larger final irrigation to refill the lysimeter after prolonged dry conditions that led to observed tree water stress (Nehemy et al., 2021). The lysimeter was also open to natural precipitation, and five rainfall events occurred during the monitoring period.

We note that the lysimeter (which contained about 500 mm of water in the soil) was also irrigated with 25 mm of isotopically labelled water ($+29.6‰$ $\delta^{18}O$ and $+256.6‰$ $\delta^{2}H$) on 16 May. The initial more enriched irrigation was used to answer other research questions described in detail by Benettin, Nehemy, Asadollahi, et al. (2021). We do not explore the tracer injection in this paper because here we focus on the difference in isotope ratios between xylem and phloem water and, critically, by the time phloem water monitoring was

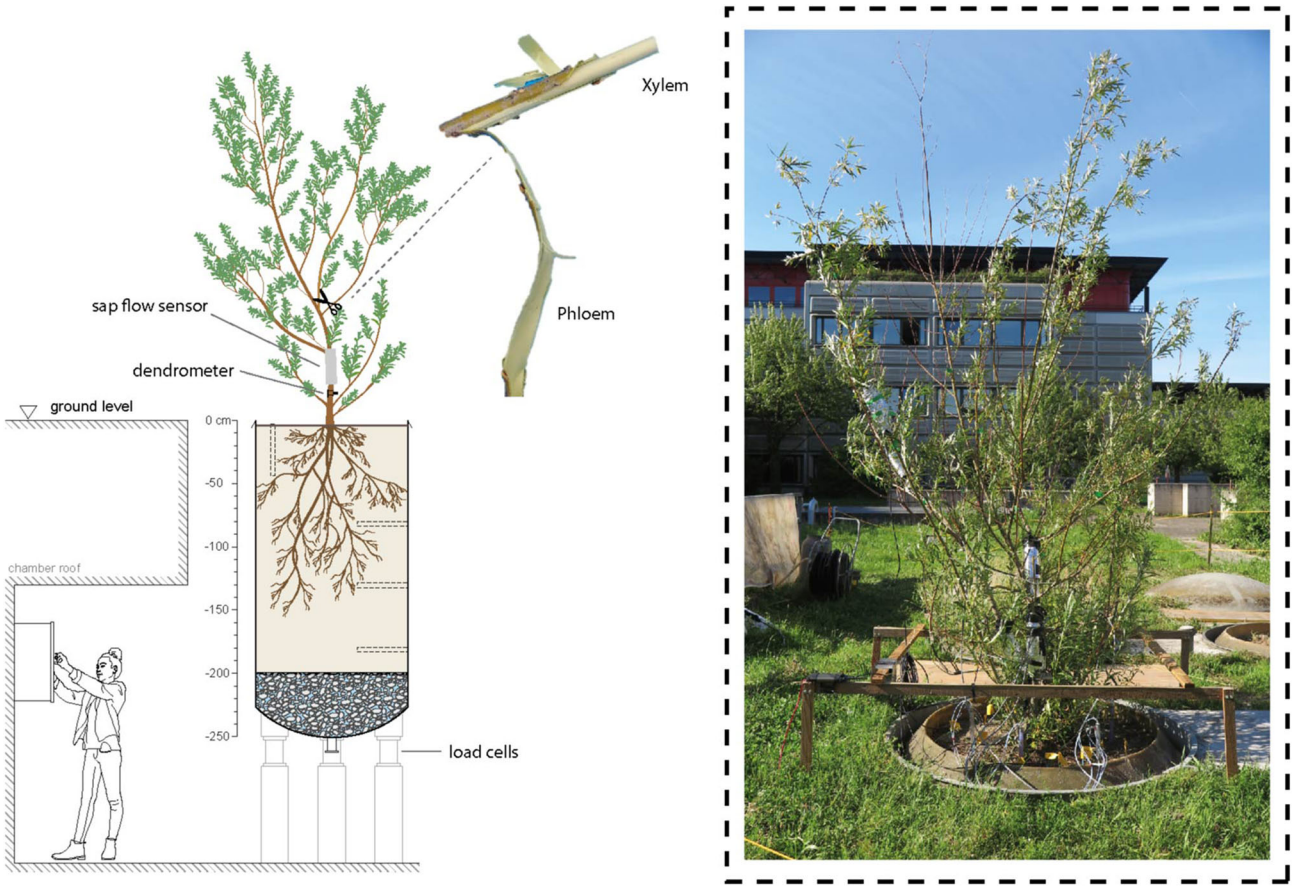


**FIGURE 1** Illustration of the experimental set-up

initiated, 17 days after the tracer injection, that tracer signature had already passed most of the root zone (at 80 cm soil depth; while root depth is up to 150 cm with greater density at surface; Nehemy et al., 2021). Additionally, the remnant tracer concentration at depth was by then about 10 times less than what was initially detected in the shallow layers as reported in Benettin, Nehemy, Asadollahi, et al. (2021). We add this note on the earlier tracer experiment for full transparency in the current analysis.

## 2.2 | Stem diameter variation and transpiration

We monitored stem diameter variations with the use of non-invasive automatic point dendrometers (Ecomatik, Germany; type DD-S, accuracy ±1.5 μm). We installed a point dendrometer at the base of each of the three main stems (that themselves originated from cuttings of the same tree; one tree showed bifurcation at the base, hence three stems) in a location free of branches. This was necessary due to the canopy architecture of the *S. viminalis*, which did not have a single, unique main stem. When the experiment started, the initial diameters of the three main stems were 29.7, 28.1 and 29.0 mm. We recorded stem diameter variation every 15 min with a datalogger (DL15, Ecomatik, Germany). These measurements provided high-resolution information of tree diameter variations, that included directional and irreversible radial growth, as well as the hydraulically reversible radial shrinkage and swelling of inner bark (Zweifel et al., 2016). We used the information from the reversible shrinkage and swelling to compute tree water deficit (TWD) and combined this with the daily use of phloem water based on the zero-growth approach developed by Zweifel et al. (2016). This approach partitions empirically the stem diameter variation into irreversible growth, and the tree–water related signal, TWD. Briefly, TWD is calculated as the difference between past maximum stem record and current record (if current record value is below last maximum diameter). We first verified that diameter variations across the main stems behaved similarly and then used the averaged value of the main stems to compute TWD (Nehemy et al., 2021). TWD indicates overall tree water status and provides information about total deficit in storages. Put simply, TWD is the difference between the radius of the fully hydrated tree and its current radius. This difference represents the total water deficit from the inner bark (Zweifel et al., 2016; Zweifel & Häsler, 2000). Thus, zero TWD indicates that tree water storage compartments are fully hydrated, and stem water potentials are close to zero. TWD values above zero represent water loss from inner bark and stem water potentials below zero. Because most of the inner bark is composed by phloem, we approximated this to deficit in phloem water storage.

We defined periods of phloem water storage contraction and expansion based on diurnal variation in diameter that reflects changes in phloem storage. Following Pfautsch, Hölttä, and Mencuccini (2015), we defined phloem expansion as periods of storage refilling, occurring from point of maximum shrinkage (after midday) to point of maximum expansion (before predawn), whereas phloem contraction shows periods of storage depletion, observed from point of maximum expansion (early morning) to point of maximum shrinkage (midday).

We computed evapotranspiration of the system (mm/h) based on measured lysimeter weight variations. Evapotranspiration was computed at 15-min resolution to reduce noise caused by wind. A more detailed description of the evapotranspiration computation and measurements can be found in Benettin, Nehemy, Asadollahi, et al. (2021). Briefly, the E/ET ratio was computed to be 10% on average; thus, T represented 90% of ET. We also installed three heat balance sensors (EXO-Skin SGA19; SGA 25, SGA 25 Dynamax, Houston, TX, USA) immediately above the dendrometers to measure sap flow rates (g/h). Because of the architecture of the *S. viminalis* trees, there were multiple branches below the sap flow sensors' height that resulted in underestimations of total transpiration. We therefore upscaled sap flow rates based on the lysimeter transpiration measurements (i.e., 90% of ET) to obtain corrected transpiration rates (mm/h) (Benettin, Nehemy, Asadollahi, et al., 2021; Nehemy et al., 2021). We note that the transpiration rates are relatively high compared to some field settings, but still within observations reported in the literature for other willows with similar planted stem density (Frédette et al., 2019), and not unanticipated given that the willows' crowns extended well beyond the 1.12 $m^2$ lysimeter bounds.

## 2.3 | Xylem and phloem water

We sampled simultaneously the isotopic composition of xylem and phloem water from 4 June to 29 June 2018. We collected xylem and phloem samples from the same branch. We selected second or third order branches that were well developed and suberized to avoid evaporative fractionation through unsuberized bark (Dawson & Ehleringer, 1993). We recorded the length, and diameter at the base and top of each branch collected, as well as from which one of the main stems it belonged to. The portion sampled from the branch was closest to the nod intersection between branch and main stem and furthest away from leaves as possible. Branches diameter ranged from 0.5 to 1.2 cm and length from 40 to 170 cm (Figure S1). After clipping the branch, we immediately separated xylem and bark. A section of bark approximately 15 cm long was peeled from the branch. Because of the very thin outer bark of this species, outer bark was not separated from the inner bark (axial parenchymal tissue, phloem, and vascular cambium) to avoid additional sample exposure to air. We refer to inner bark as phloem since most of the inner bark water volume is stored in the phloem (Lazzarin et al., 2019; Zweifel & Häsler, 2000), similarly to previous studies (Adar et al., 1995; Wang et al., 2021). We stored xylem and phloem in separated 12 ml gas-tight glass vials (Exetainer®, Labco, Lampeter, UK), wrapped all vials with Parafilm® and then stored in a refrigerator at 4°C.

The frequency of sampling varied throughout the 24 xylem–phloem sampling days. We sampled every other day from 4 June to 10 June and then less frequently from 14 June to 21 June. Then, we introduced a period from 21 to 26 June where we made higher frequency measurements along the stem diurnal cycle to observe high-

resolution xylem and phloem isotopic composition variability. The latter marks the transition from driest period observed in the lysimeter along with increase in tree water stress to a well-watered condition, followed by tree water status recovery (Nehemy et al., 2021). From 21 to 26 June, we collected samples at predawn before transpiration started where the tree had the highest storage volume for the day, at the beginning of transpiration, during midday and end of the day when phloem water storage was being refilled.

We also sampled precipitation and soil water isotopic composition during the experiment. We collected bulk, event precipitation using a rainfall collector placed 2 m away from the trees. Precipitation was bottled for isotope analysis immediately after the event or in the next morning when it occurred overnight. We collected soil samples for laboratory water extraction every 4 days across different depths in the soil profile (10, 25, 50, 80 and 150 cm). We sampled soil using a small auger (diameter 3 cm) and access ports along the side of the lysimeter. The isotopic values of soil water are used to understand the range distribution of soil water in dual-isotope space in comparison with isotopic values of phloem and xylem water.

We conducted water extraction and subsequent isotopic analysis at the Hillslope Hydrology Laboratory, Canada. We extracted xylem and phloem water using a cryogenic vacuum distillation system following (Koeniger et al., 2011). We weighed vials prior and after cryogenic extraction, then oven dried samples at 100°C for 24 h and reweighed to ensure cryogenic water extraction efficiency (median extraction efficiency = 100%; IQR = 0). Each water sample was filtered through a 0.45 μm and stored in 2 ml vials with septum screw caps for isotopic analysis. We analysed the isotopic composition of xylem and phloem using isotope ratio mass spectrometry (IRMS) to avoid spectral contamination. Our IRMS laboratory precision for this project was ±0.12‰ on $\delta^{18}O$ and ±0.81‰ on $\delta^{2}H$ ($n = 8$; water standards). $\delta^{18}O$ and $\delta^{2}H$ values are expressed in delta notation relative to the international standard V-SMOW.

## 2.4 | Allometric measurements of sapwood and storage

Following the completion of our experiment, we cut down the main stems on 29 June to quantify total tree water storage. We measured individual branches and main stem diameters at the base ($g_i$; radius = $r_i$) and top ($g_{i+1}$; radius = $r_{i+1}$) and the length ($l$) between those two points. We used Smalian's formula to quantify volume ($v_i$) of each individual branch and main stems:

$$v_i = \frac{\pi r_i^2 + \pi r_{i+1}^2}{2} \times l, \quad (1)$$

$$V = \sum_{i=1}^{n} (v_i + v_{i+1} + \ldots + v_{i+n}). \quad (2)$$

Then, we obtained total tree volume ($V$) by adding individual volume of each branch and main stems ($v_i$):

We selected a subsample of branches ($n = 10$) of different diameters to assess the relationship between bark thickness and branch diameter. Visible inspection of the outer bark of *S. viminalis* showed it to be thin, and thus, we assumed that most of the thickness represented the phloem. We used this information to develop a linear relationship between branch diameter and phloem thickness. Using this linear relationship (adjusted $R^2 = 0.89$; $p < 0.001$; Figure S2), we were able to calculate the phloem thickness of all individual branches and stems collected. We then obtained diameter information of each branch without phloem by subtracting phloem thickness and obtaining wood diameter only ($g_{wib}$).

We used the branch diameter information without phloem ($g_{wib}$) to quantify total sapwood volume only ($V_{sap}$). This was done by using Smalian's formula 1 to obtain sapwood volume ($V_{sap}$) by inputting diameter of branches and stems without inner bark ($g_{wib}$). We then obtained the total phloem storage volume ($V_{stg}$) by subtracting $V_{sap}$ from total tree volume ($V$).

We used $V_{stg}$ to upscale daily changes in stem diameter to the whole tree and compute daily contribution of phloem water to total transpiration. Because TWD provides information about diameter variability related to changes in tree water storage only (i.e., without growth), we first computed daily TWD amplitudes to obtain total daily use of phloem water storages (diameter, μm):

$$\Delta\text{Storage}_{\text{phloem}} = \text{TWD}_{\text{max}} - \text{TWD}_{\text{min}}. \quad (3)$$

When we cut down the *S. viminalis*, tree water status showed that trees were not in deficit (Nehemy et al., 2021), so we assume that phloem water storage was closest to its maximum capacity at this point. Thus, we used the calculated total phloem volume ($V_{stg}$) to upscale observed daily changes in storage to the whole tree by approximating measured daily variation in $\Delta\text{Storage}_{\text{phloem}}$ and recomputing daily $V_{stg}$ based on daily changes in stem diameter. This is a similar approach to the method described by Génard et al. (2001) but adapted to the architecture of the trees in this experiment and available measurements. Measurements of $\Delta\text{Storage}_{\text{phloem}}$ provides an estimation of how much phloem water is contributing to daily transpiration, which enable us to understand the relationship between phloem water use and variability in phloem water isotope values.

## 2.5 | Data analysis

We performed all statistical analysis in R 3.6.3 (R Core Team, 2020). We computed the isotopic $\delta^{2}H$ and $\delta^{18}O$ difference between phloem and xylem pools (expressed at per mil, ‰) as

$$\Delta_{\text{phloem - xylem}} = \delta_{\text{phloem}} - \delta_{\text{xylem}}, \quad (4)$$

where $\delta_{\text{phloem}}$ and $\delta_{\text{xylem}}$ stand for the hydrogen or oxygen isotopic signatures of phloem and xylem, respectively. Negative values show that phloem is more depleted in heavier isotopes than xylem, while positive values indicate that phloem is more enriched than xylem

water. We used the Wilcoxon signed-rank test to assess if observed differences in isotopic values of phloem and xylem water were significantly different. We used a non-parametric test because phloem and xylem water stable isotope data were found to be non-normally distributed. We computed means, standard error and standard deviation of phloem and xylem $\delta^{18}O$ and $\delta^{2}H$ values across all samples and within the *S. viminalis*, using samples collected on 29 June. We further used a non-parametric Spearman rank correlation to describe the temporal relationship between phloem and xylem water during the experiment.

We computed $\Delta_{phloem-xylem}$ and means across periods of diurnal cycle (i.e., night-time, predawn, morning and midday) to assess the influence of phloem water storage refilling and depletion on the isotopic values of phloem and xylem water. We investigated the relationship between $\Delta_{phloem-xylem}$ and daily use of phloem water storage ($\Delta Storage_{phloem}$) and transpiration rates using general linear models. We further investigated the influence of tree water status on $\Delta_{phloem-xylem}$ by assessing whether the isotopic composition of phloem and xylem water was statistically different prior and after the recovery of tree water status. We used the TWD to determine the recovery of tree water status (TWD = 0 at night) and Wilcoxon signed-rank test for statistical testing of significance. The significance level for all statistical tests was set to the 95% confidence interval.

## 3 | RESULTS

### 3.1 | Phloem and xylem isotopic composition

Phloem and xylem isotopic composition ($\delta^{18}O$ and $\delta^{2}H$) were significantly distinct from one another across the sampling period (Wilcoxon signed-rank test, $p < 0.001$). The average phloem water isotopic composition was −9.04‰ ± 0.77 (mean ± 1 SD) for $\delta^{18}O$ and −74.26‰ ± 5.77 for $\delta^{2}H$ ($n = 33$), and the average xylem water was −8.53‰ ± 0.85 for $\delta^{18}O$ and −73.10‰ ± 6.08 for $\delta^{2}H$ ($n = 40$). Our data show that xylem water plotted further away from the local meteoric water line (LMWL) than phloem water (Figure 2) and was systematically more enriched in heavy isotopes than was phloem water (Figure 3 and Table 1). In dual-isotope space, both phloem and xylem water plotted within the range of soil water isotopic composition (Figure 2). The soil water isotopic composition in this study broadly reflects winter rainfall inputs (as shown by Benettin, Nehemy, Cernusak, et al., 2021).

Temporal variability in the $\Delta_{phloem-xylem}$ was smaller than within tree variability (Table 1). Samples collected by destructive sampling at midday on 29 June showed smaller differences than overall temporal differences. The mean difference in isotopic composition between phloem and xylem water within all measured samples ($n = 33$) was −0.63‰ ± 0.10 (mean ± SE) for $\delta^{18}O$ and −2.05 ± 0.49‰ for $\delta^{2}H$. The observed difference is greater than isotopic measurements analytical precision. The largest difference between phloem and xylem water was recorded on 21 June at night (21:00), with a $\delta^{18}O$ difference of −1.73‰ and $\delta^{2}H$ difference of −6.89‰ during a period of tree water stress (TWD > 0). The smallest difference in $\delta^{2}H$ was −0.85‰ observed before dawn (4:15) on 22 June, and the smallest difference in $\delta^{18}O$ was −0.06‰ on 23 June at midday (13:00) after irrigation and recovery of tree water status. Except for a few events with positive $\Delta_{phloem-xylem}$ values that were observed in days when TWD calculations did not indicate TWD, the $\Delta_{phloem-xylem}$ were negative, indicating a general depletion of heavy isotopes of phloem water in relation to xylem water. Although phloem and xylem isotopic values were statistically distinct, xylem and phloem water isotope ratios

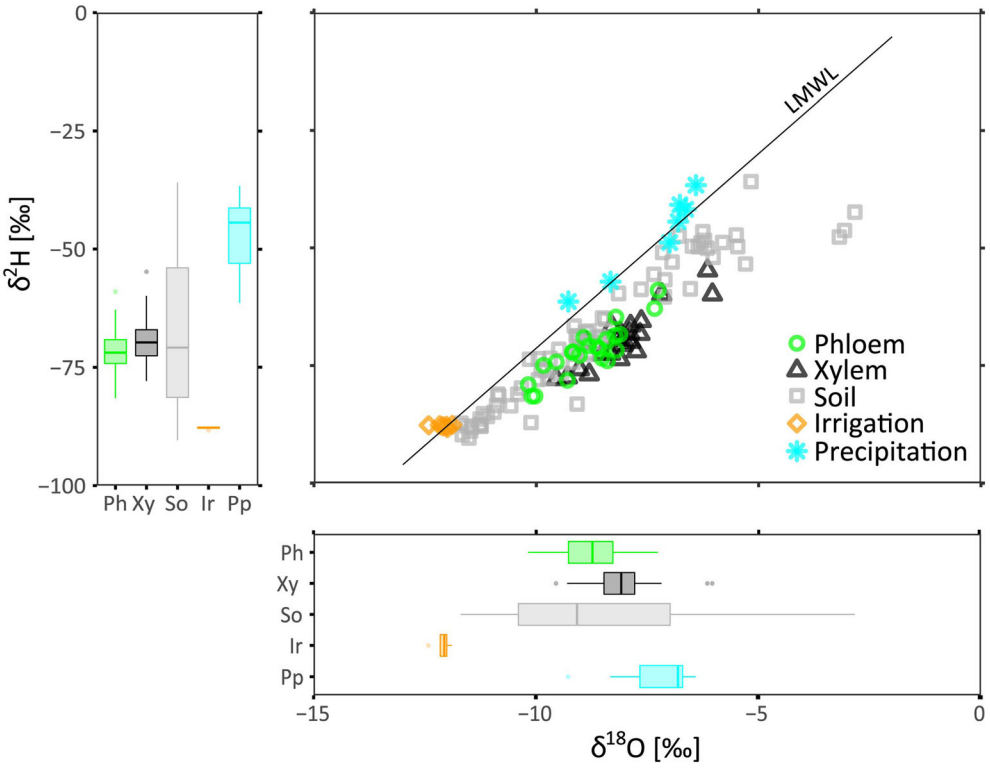


**FIGURE 2** Dual-isotope plot showing isotopic composition of water in phloem (Ph), xylem (Xy) and soil (So), as well as of irrigation water (Ir) and precipitation (Pp). Samples were collected between 4 and 29 June, prior to tree cut down. Line shows the local meteoric water line (LMWL)

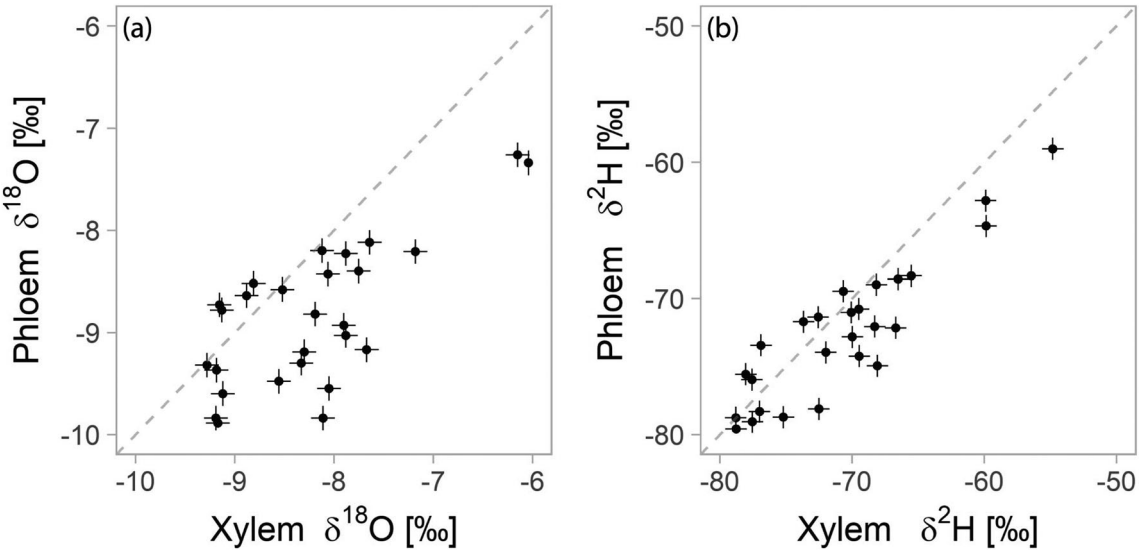


**FIGURE 3** Phloem water isotope composition plotted against xylem water isotopic composition. Panels (a) and (b) show $\delta^{18}O$ and $\delta^{2}H$, respectively. The dashed line is the 1:1 line. Error bars show measured analytical precision

**TABLE 1** Isotopic difference between phloem and xylem water ($\Delta_{phloem-xylem}$) in *Salix viminalis* calculated contemporaneously

| | $\delta^{18}O$ | SD | $\delta^{2}H$ | SD | *n* |
|---|---|---|---|---|---|
| All samples | −0.63 | ±0.59 | −2.05 | ±2.80 | 33 |
| Temporal variability | −0.74 | ±0.53 | −2.47 | ±2.65 | 23 |
| Within tree variability | −0.37 | ±0.60 | −1.17 | ±2.89 | 11 |

*Note*: *Temporal variability* includes samples from 4 June to 29 June prior tree cut down. *Within tree variability* includes only samples collected during the afternoon of 29 June during tree destructive sampling.

covaried, showing strong correlations in both $\delta^{18}O$ ($r = 0.79$) and $\delta^{2}H$ ($r = 0.90$) throughout the experiment (Figure 4).

### 3.2 | Daily use of phloem water

The $\delta^{2}H$ and $\delta^{18}O$ difference between phloem and xylem was statistically shown to increase with the increase in daily use of phloem water ($\Delta Storage_{phloem}$), while $\delta^{18}O$ difference between phloem and xylem increases with the increase in transpiration rates (Figure 5). The differences ($\Delta_{phloem-xylem}$) become more negative, indicating that phloem becomes more depleted in heavier isotopes than xylem water with increasing transpiration and phloem water use.

The *S. viminalis* transpired on average 11.8 mm ± 2.98 of water per day from 4 to 28 June. Transpiration rates showed a small increase from 14 to 21 June, while phloem water storage contribution to transpiration decreased during this period (Figure 6c). Despite the small contribution of phloem water to daily transpiration (<1%), there is variable difference between phloem and xylem water (Figure 6d,e).

### 3.3 | Phloem water storage refilling and tree water status

We observed variability in $\Delta_{phloem-xylem}$ throughout the diurnal cycle (e.g., night-time, predawn, morning and midday) (Figure 7). Overall, the largest difference in isotopic composition between phloem and xylem was observed during phloem refilling in the evenings (shaded blue) (Figure 7b–d), whereas differences decreased during predawn (4–5 AM) and morning (8–10 AM) (Table 2). Phloem refilling was usually observed from 15:00 to 2:00 indicated by the stem expansion (Figure 7d), along with decreasing transpiration rates (Figure 7e). During predawn and early morning, TWD was closer to zero (Figure 7d) showing that phloem water storage pools were replenished. At midday, stem shows maximum TWD and stem shrinkage and transpiration rates (Figure 7e).

Besides the subdaily variability between phloem and xylem, this transition period, from TWD to the recovery of tree water status, showed that the largest difference between phloem and xylem isotopic composition occurred in TWD conditions. Overall, phloem and xylem were distinct in both, $\delta^{18}O$ ($p < 0.01$) and $\delta^{2}H$ ($p < 0.05$) prior to irrigation during TWD conditions (TWD always >0; not recovering at night), whereas phloem and xylem were not distinct in $\delta^{18}O$ and $\delta^{2}H$ ($p > 0.05$) after irrigation and recovery of tree water status (Figure 7b–d).

## 4 | DISCUSSION

It is well known that the radial water flow from phloem to xylem is important to maintain tree water transport integrity (Pfautsch, Hölttä, & Mencuccini, 2015; Steppe et al., 2006; Zweifel et al., 2001).

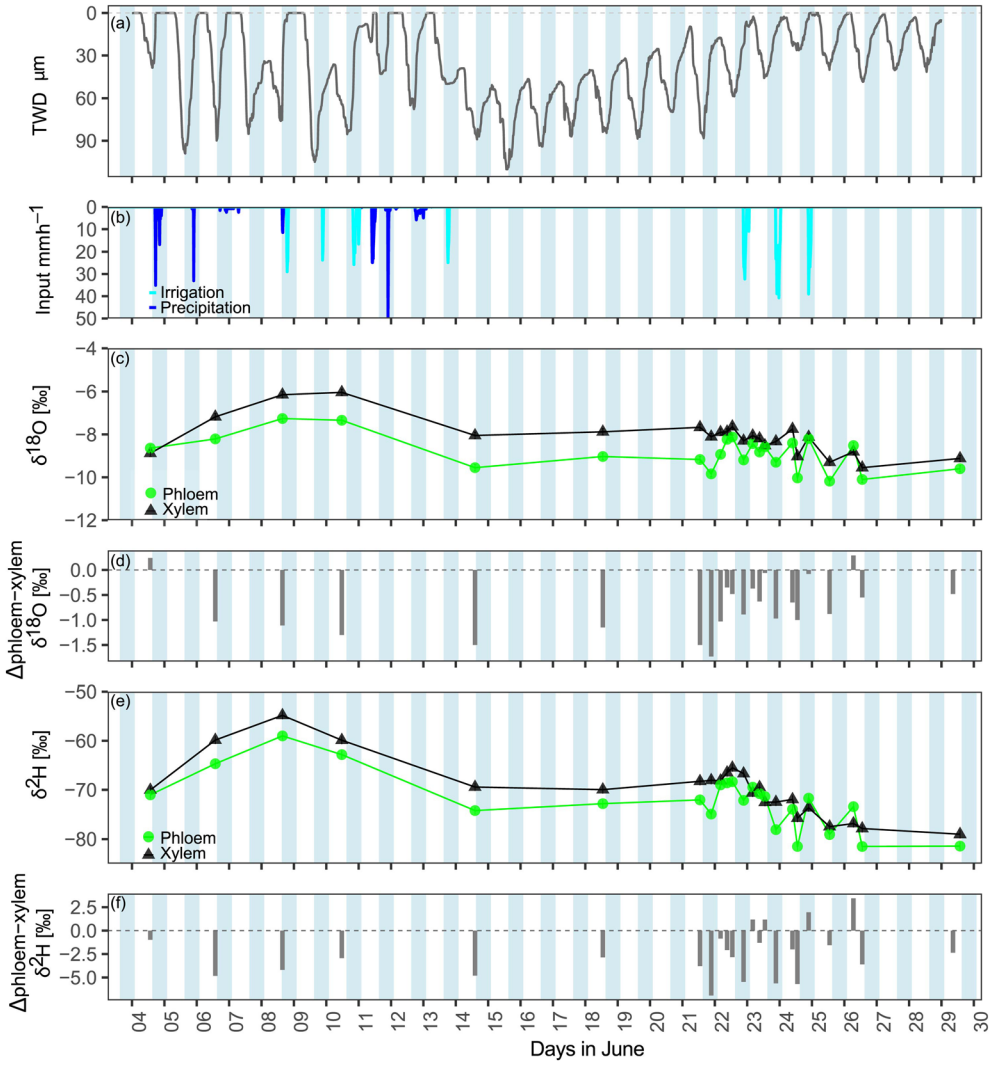


**FIGURE 4** Phloem and xylem water isotopic composition during the experiment. Panel (a) shows tree water deficit (TWD). Panel (b) shows irrigation and inputs. Panels (c) and (e) show $\delta^{18}O$ and $\delta^{2}H$ phloem and xylem water isotopic composition, respectively. Panels (d) and (f) show the difference between phloem and xylem $\delta^{18}O$ and $\delta^{2}H$ values ($\Delta_{phloem-xylem}$), respectively. Negative values show that phloem is more depleted in heavier isotopes than xylem, while positive values indicate that phloem is more enriched than xylem water. Blue bands indicate 15:00 to 2:00, the period we found to correspond with phloem water refilling

However, it is unknown how this daily phloem−xylem radial flux and the contribution of phloem storage affects the isotopic composition of xylem water and how changes in tree water status and daily use of phloem water might affect the isotopic composition of these pools. To the best of our knowledge, our study is the first to report the temporal evolution of $\delta^{18}O$ and $\delta^{2}H$ in both phloem and xylem water at daily and subdaily time resolutions.

## 4.1 | Phloem and xylem water isotopic composition

Despite the seemingly small phloem contribution to total daily transpiration (Figure 6), the use of stable isotopes of hydrogen and oxygen in our investigation yielded new insights into xylem and phloem water relations at daily and subdaily resolutions. For instance, our data showed that phloem water was less enriched in heavy isotopes relative to xylem water (hereafter termed 'enriched' when referring to 'enriched in heavy isotopes'). Despite the increasing number of studies investigating oxygen stable isotope composition of phloem organic matter in tree species (e.g., Bögelein et al., 2019; Gessler et al., 2007; Lehmann et al., 2018; Treydte et al., 2014), the study of phloem water isotopic composition in relation to xylem water is incomplete.

Only few studies that we are aware of have previously reported isotopic signatures of phloem water in relation to xylem in trees (Table 3). Our results contradict early studies that indicated that phloem water tended to be more enriched in relation to xylem water (Adar et al., 1995; Cernusak et al., 2005; Wershaw et al. 1970). The previously reported small enrichment in phloem water in relation to xylem was explained by possible enriched leaf water migrating down to phloem. Additionally, phloem water enrichment has been previously reported during periods of high relative humidity (i.e., leaf wetting or fog events). Wang et al. (2021) showed that leaf water enrichment is seen in phloem water in relation to xylem with fog. However, in this same experiment, Wang et al. (2021) also observed that during natural conditions (prior to and 2 days after artificial fog injection; Table 3), phloem water was more depleted in heavy isotopes in relation to xylem water (hereafter termed 'depleted' when referring to 'depleted in heavy isotopes'). This was more evident for $\delta^{2}H$ and in the natural dry forest

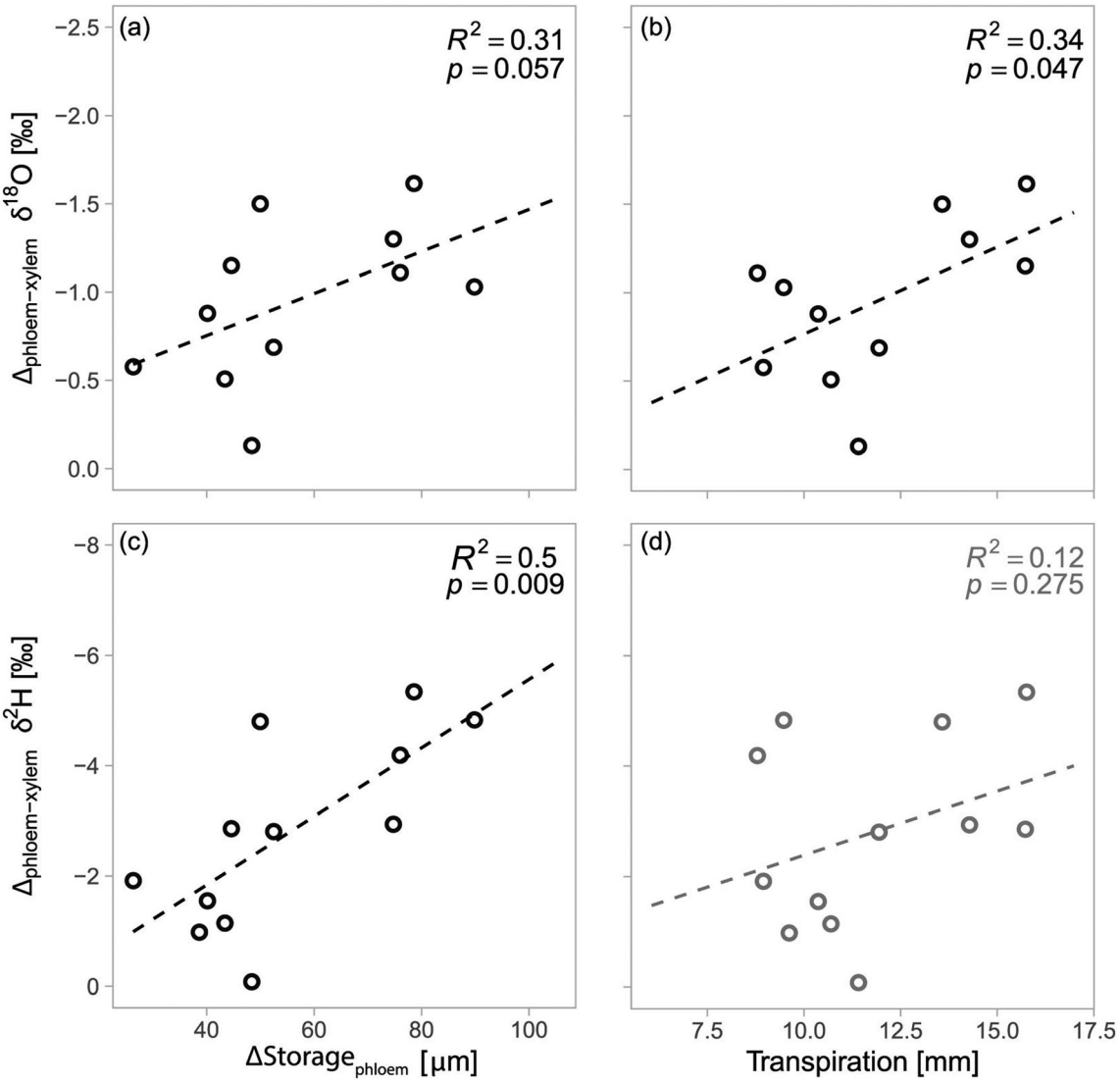


**FIGURE 5** Relationship between $\delta^{18}O$ and $\delta^{2}H$ difference ($\Delta_{phloem-xylem}$) between phloem and xylem water and daily use of (a,c) phloem water storage ($\Delta Storage_{phloem}$) and (b,d) transpiration

in comparison to the long-term irrigated treatment (Table 3). Our work supports this recent evidence by Wang et al. (2021) with more negative isotope values in phloem water and larger $\Delta_{phloem-xylem}$ during TWD conditions when compared to values after irrigation and recovery of tree water status (Table 3; Figure 7). Recently, Treydte et al. (2021) investigating water movement in two species of Eucalyptus in Australia (*Eucalyptus tereticornis* and *Eucalyptus sideroxylon*) also showed that phloem water was more depleted than xylem in both sapwood and heartwood consistent with our overall results. Our results along with recent literature evidence show that the early assumption that phloem is more enriched than xylem water because of possible leaf water enrichment might not be widespread, and $\Delta_{phloem-xylem}$ might also be dependent on tree water status.

## 4.2 | A proposed phloem water recharge mechanism

Despite our finding that phloem water was more isotopically depleted in relation to xylem water, we observed a strong correlation in the temporal variations between phloem and xylem for both $\delta^{18}O$ and $\delta^{2}H$ (Figure 2). Such evidence suggests that these two pools are hydraulically connected. We observed daily stem shrinkage during the day and expansion at night, supporting that passive water transport between xylem and phloem tissues occurs, driven by the transpiration-induced diurnal variation of water potential. These results support previous theory and empirical measurements that stressed the tight hydraulic coupling between xylem and phloem (Lazzarin et al., 2019; Pfautsch, Hölttä, & Mencuccini, 2015; Sevanto

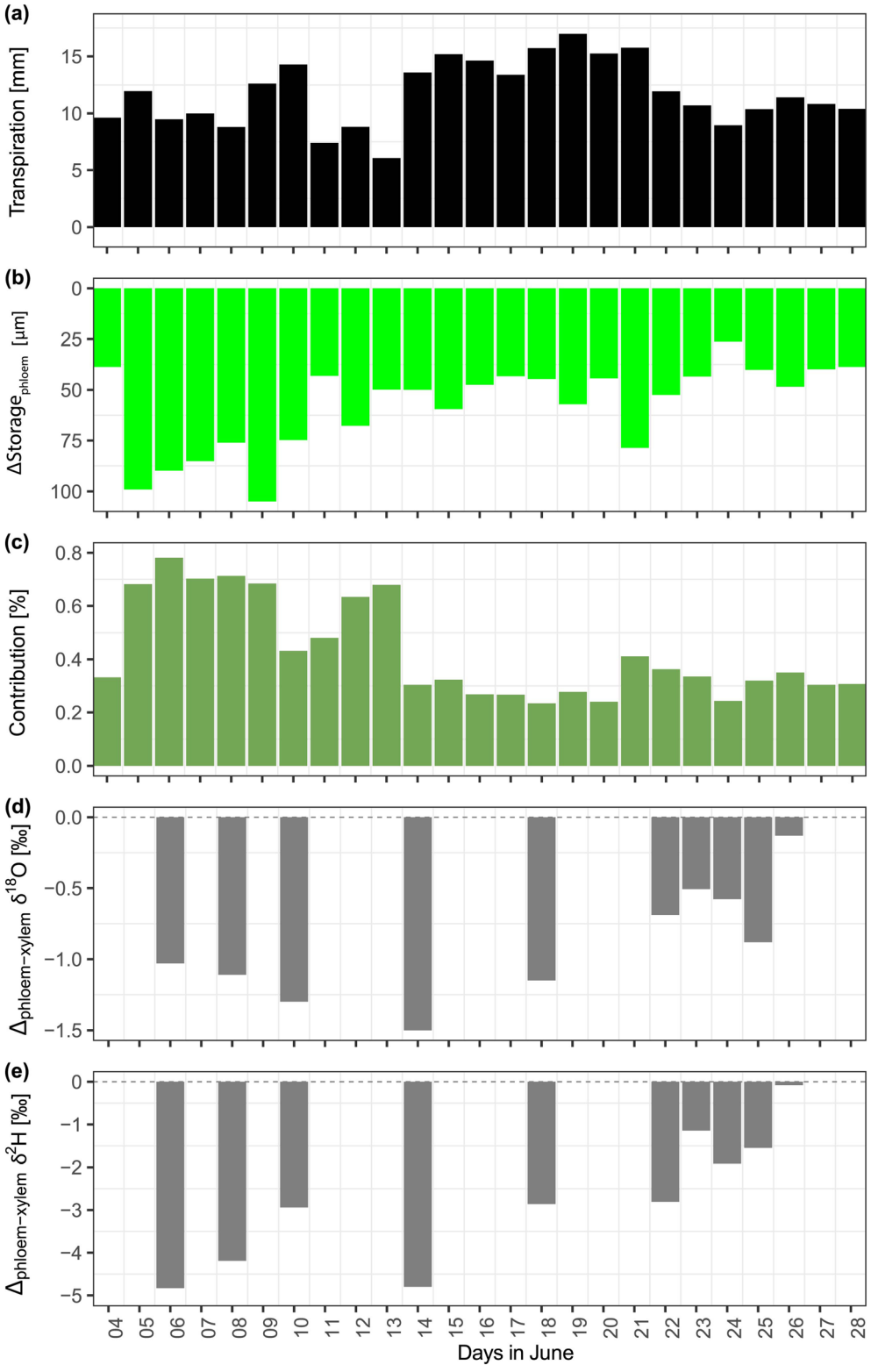


**FIGURE 6** Daily contribution of phloem storage to transpiration. Panel (a) shows daily amount of transpiration. Panel (b) shows phloem water storage ($\Delta Storage_{phloem}$). Panel (c) shows percentage of phloem water contribution to transpiration (note: *y* axis indicate 0% to 0.8%, not 80%), inferred hydrometrically. Panels (c) and (d) show $\delta^{18}O$ and $\delta^{2}H$ average daily difference between phloem and xylem, respectively

et al., 2011; Steppe et al., 2012; Zweifel et al., 2001). Phloem and xylem are radially connected by parenchyma rays (Pfautsch, Renard, et al., 2015), and the bidirectional-radial water transport between these interconnected pools occurs as a result of the water potential gradient between phloem and xylem (Pfautsch, Hölttä, & Mencuccini, 2015; Sevanto et al., 2011; Steppe et al., 2012, 2006).

Sevanto et al. (2011) showed empirically that the radial transport of water between xylem and phloem is determined by the radial conductance between them. They suggested that values obtained for radial conductance between phloem and xylem are within water conductance values reported for aquaporins transport. Thus, the cell-to-cell pathway modulated by aquaporins has been proposed as the main

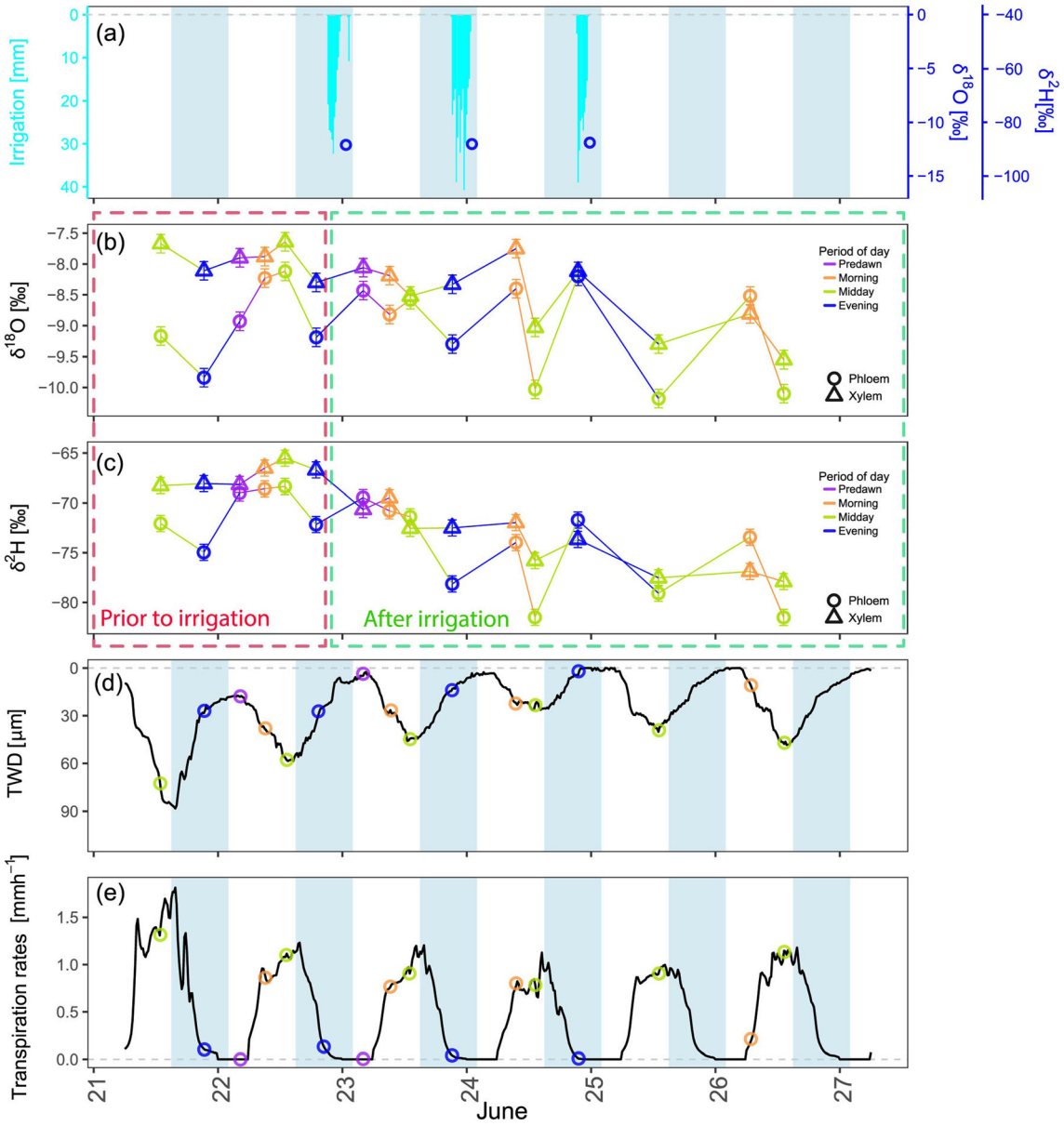


**FIGURE 7** Isotopic composition of phloem and xylem at a subdaily resolution along with tree water deficit (TWD) and transpiration rates. Panel (a) shows irrigation volume (cyan) along with $\delta^{18}O$ and $\delta^{2}H$ isotopic composition of irrigation (dark blue). Panels (b) and (c) show $\delta^{18}O$ and $\delta^{2}H$ phloem and xylem water during the diurnal cycle, respectively. Panel (d) shows TWD. Panel (e) shows transpiration rates. Blue bands indicate main period of phloem water refilling between 15:00 to 2:00 of each day. Panels indicate the subdaily variability in isotopic composition between phloem and xylem relative to changes in stem diameter and transpiration rates

**TABLE 2** Mean phloem and $\delta^{18}O$ and $\delta^{2}H$ values and differences ($\Delta_{phloem-xylem}$) in bold reported per period of day and stem diameter status

| Period of day | Stem diameter | Mean $\delta^{18}O$ phloem | Mean $\delta^{18}O$ xylem | $\Delta_{phloem-xylem}$ $\Delta^{18}O$ | Mean $\delta^{2}H$ phloem | Mean $\delta^{2}H$ xylem | $\Delta_{phloem-xylem}$ $\Delta^{2}H$ | *n* |
|---|---|---|---|---|---|---|---|---|
| Evening | Expansion (storage refilling) | −9.25 | −8.21 | **−0.91** | −74.2 | −70.2 | **−4** | 4 |
| Predawn–morning | Max. expansion–contraction | −8.56 | −8.1 | **−0.46** | −70.9 | −70.6 | **−0.3** | 6 |
| Midday | Max. contraction (storage depleted) | −9.36 | −8.77 | **−0.74** | −75.6 | −72.9 | **−2.7** | 6 |

**TABLE 3** Mean phloem and $\delta^{18}O$ and $\delta^{2}H$ values and differences ($\Delta_{phloem-xylem}$) (in bold) reported per species in this study and in the literature

| Species | Location | Obs | Tree segment | Mean $\delta^{18}O$ phloem | Mean $\delta^{18}O$ xylem | $\Delta_{phloem-xylem}$ $_{\Delta}{}^{18}O$ | Mean $\delta^{2}H$ phloem | Mean $\delta^{2}H$ xylem | $\Delta_{phloem-xylem}$ $\Delta^{2}H$ | Source |
|---|---|---|---|---|---|---|---|---|---|---|
| *Populus sargentii* | Colorado, USA | Summer ($n = 10$) | Stem | - | - | - | −9.69 | −9.07 | **−0.09** | Wershaw et al. (1970)[a] |
| *Salix* sp. | Colorado, USA | Summer ($n = 3$) | Stem | - | - | - | −12.47 | −12.50 | **0.03** | Wershaw et al. (1970) |
| *Alnus rubra* | Colorado, USA | Summer ($n = 1$) | Stem | - | - | - | −5.1 | −5.1 | **0** | Wershaw et al. (1970) |
| *Tamarix* spp. | Israel | Summer ($n = 2$) | Stem | 10.30 | −2.93 | **13.23** | - | - | - | Adar et al. (1995) |
| *Tamarix* spp. | Israel | Summer ($n = 2$) | Roots | 10.78 | −4.37 | **14.125** | - | - | - | Adar et al. (1995) |
| *Eucalyptus globulus* | Australia | Nov–Dec, Mar ($n = 98$) | Stem/branch | - | - | **0.5–0.8** | - | - | - | Cernusak et al. (2005) |
| *E. globulus* | Australia | Dec ($n = 63$) | Stem/branch | −3.83 | −4.62 | **0.78** | - | - | - | Cernusak et al. (2005)[b] |
| *Pinus sylvestris* | Switzerland | Control—dry treatment ($n = 15$) | Twig | −5.57 | −4.47 | **−1.10** | −61.07 | −47.35 | **−13.72** | Wang et al. (2021)[c] |
| *P. sylvestris* | Switzerland | Irrigated—wet treatment ($n = 15$) | Twig | −8.02 | −7.06 | **−0.96** | −77.77 | −72.2 | **−5.55** | Wang et al. (2021) |
| *Eucalyptus sideroxylon* | Australia | Summer ($n = 1$) | Stem | −5.20 | −2.60 | **−2.60** | −39.50 | −24.4 | **−15.10** | Treydte et al. (2021)[d] |
| *Eucalyptus tereticornis* | Australia | Summer ($n = 1$) | Stem | −5.00 | −2.60 | **−2.40** | −35.80 | −19.6 | **−16.20** | Treydte et al. (2021) |
| *Salix viminalis* | Switzerland | Overall ($n = 33$) | Branch | −9.04 | −8.41 | **−0.63** | −74.26 | −72.22 | **−2.05** | This study |
| *S. viminalis* | Switzerland | Subdaily, dry conditions ($n = 6$) | Branch | −8.91 | −7.92 | **−0.99** | −70.90 | −67.2 | **−3.70** | This study |
| *S. viminalis* | Switzerland | Subdaily, wet conditions ($n = 11$) | Branch | −9.11 | −8.62 | **−0.49** | −75.70 | −74.40 | **−1.30** | This study |

[a]Summary in tabs. 1–3.
[b]Summary in tab. 7.
[c]This analysis and $\delta^{2}H$ data are not published in Wang et al. (2021). Data are summarized here.
[d]Data from neighbour trees with natural isotopic abundance (i.e., not influenced by artificial tracer enrichment). Xylem water reflects sapwood and not heartwood.

pathway for water movement between xylem and phloem by Sevanto et al. (2011). Steppe et al. (2012) showed that radial water transport is variable and that the variability in radial conductance occurs as result in changes in water potential. They further hypothesized that the radial water transport can occur via two parallel forms of cell-to-cell modulation by aquaporins and by apoplastic transport. Steppe et al. (2012) showed that the radial flow is modulated by aquaporins when transpiration demands are high; alternatively, apoplastic transport takes place when hydraulic demands are low, for example, at predawn and early morning. A fluorescent tracing experiment showed that the radial flow of water between phloem and xylem occurred mostly through the symplastic pathway (i.e., rays), indicating that it may be modulated by aquaporins (Pfautsch, Renard, et al., 2015). Stanfield et al. (2017) showed for the first time the existence of aquaporin in phloem supporting their participation in xylem–phloem water transport. Aquaporins have been previously shown to modulate water transport in leaves and roots at times of high hydraulic demands (e.g., Aroca et al., 2005; Cochard et al., 2007; Martre et al., 2001) and are associated with fast water transport (Kozono et al., 2002). Aquaporins are also shown to be present in parenchyma cells associated with xylem vessels, including rays (i.e., three dimensional symplastic continuum of parenchyma cells that connects xylem and phloem) (Secchi et al., 2017). Secchi et al. (2017) showed the importance of aquaporins in parenchyma cells during water stress recovery.

The evidence of aquaporin modulation of water movement between xylem and phloem in trees is especially important for stable isotope investigations of tree water use. Previous reports of more fractionated xylem water signatures in trees have been related to cases where aquaporins are likely the main channel for water transport during water uptake, that is, instead of the apoplastic pathway (Ellsworth & Williams, 2007; Poca et al., 2019). The isotope fractionation of water during aquaporin transport has been proposed previously (Poca et al., 2019; L. Zhao et al., 2016) based on experimental evidence reporting a lower osmotic permeability of $^{2}H_{2}{}^{16}O$ compared to $^{1}H_{2}{}^{16}O$ (Mamonov et al., 2007). Mamonov et al. (2007) showed that lower permeability of $^{2}H_{2}{}^{16}O$ compared to $^{1}H_{2}{}^{16}O$ occurs because of the low self-diffusion coefficient of $^{2}H_{2}{}^{16}O$ ($8.5 \times 10^{-14}$ $cm^3/s$/pore) and higher viscosity when compared to $^{1}H_{2}{}^{16}O$ ($10.0 \times 10^{-14}$ $cm^3/s$/pore). The water transport through aquaporins occurs in a single-file chain of water molecules through the small aquaporin pore (2.8 Å in diameter), similar in diameter to the water molecule itself (Kozono et al., 2002). The aquaporin pore is also lined with a hydrophobic surface that prevents the water molecule from forming H bonds with an adjacent water molecule during transport (Murata et al., 2000). When water transport is modulated by aquaporins, the water 'squeezes' through the narrow pore resulting in a reorientation of the water molecule structure involving a fine-tuned dipole inversion and rupturing of H bonds (Groot & Grubmüller, 2001; Hub et al., 2009; Murata et al., 2000; Tajkhorshid et al., 2002). When the H-bond rupture occurs, the pore offers a 'replacement' interaction, and the water molecule forms a H bond with the pore surface (Groot & Grubmüller, 2001; Murata et al., 2000; Tajkhorshid et al., 2002). While there is limited evidence of fractionation through aquaporins, previous studies have also reported isotope fractionation associated with water transport and/or confinement in small pore spaces similar to aquaporins (e.g., Chen et al., 2016; Lin et al., 2018; Lin & Horita, 2016; Richard et al., 2007). The isotope fractionation of interfacial and/or confined water seems to occur because the decrease in pore size results in an increasing distortion of the tetrahedral water structure and rupture of H bonds (Smirnov et al., 2000), similar to what is described in the literature for aquaporin water transport. The water molecule adsorbed to small pores has a stronger H bond to the pore surface, and a weaker H bond with adjacent molecules, which affects molecular orientation (Lin et al., 2018). The fractionation effect of adsorbed water is also larger on hydrogen than on oxygen (Lin et al., 2018). Thus, we hypothesize that the preferential transport between xylem and phloem through aquaporins' small pore space and higher observed diffusion of lighter isotopes could possibly explain the more depleted isotopic composition of phloem water in relation to xylem observed in this study. This is especially the case during periods of phloem water refilling and TWD when aquaporin is likely to be prevalent water transport pathway.

We propose that when water enters the xylem in the roots and moves into the phloem via aquaporins, phloem tissue is preferentially refilled by water with lighter isotopes ($^{1}H$ and $^{16}O$), and xylem water becomes slightly enriched ($^{2}H$ and $^{18}O$) deviating from the LMWL. We observed larger difference during phloem water refilling when stem radius started to expand early in the evening, throughout the night. How significantly enriched in heavy isotopes xylem water becomes will likely depend on the volume of water necessary to refill the phloem storage (Figure 5), integrated over time, and the relative size of sapwood water storages. This process will be also dependent on the hydraulic demands in time of recharge that will determine the pathway type, that is, via apoplastic, where bulk flow dominates water transport, or via transmembrane, where water diffusion across cell membrane is modulated by aquaporins. Our data support this refilling fractionation mechanism via the significant linear relationship that we found between daily use of phloem water storage and the isotopic difference between phloem and xylem (Figure 5). When $\Delta Storage_{phloem}$ values were integrated over the course of a day, our data indicate that the $\Delta_{phloem-xylem}$ increases with the increased use of phloem storage, likely because more water needs to be refilled at night. Although this relationship explains 50% or less of observed variability in the difference between phloem and xylem, a more complete predictor will likely also include the change in radial hydraulic conductance between phloem and xylem.

The overall differences between phloem and xylem decreased after irrigation on the night of 22 June and recovery of tree water status. Increase in water availability via irrigation and recovery of tree water status on the night of 23 June (Figure 7) may have resulted in decreased water potential gradients in the xylem and phloem, leading to a prevalence of apoplast transport over aquaporins diffusion as proposed by Steppe et al. (2012). During low hydraulic demands, Steppe et al. (2012) have proposed that phloem and xylem water transport would occur via the apoplast. During this period of higher water availability and recovery of tree water status at subdaily

resolution, differences between xylem and phloem were small (Figure 7). This is also supported by Wang et al. (2021) experiment that showed smaller isotopic differences between phloem and xylem water in the irrigated treatment in comparison to the drier treatment under natural conditions (Table 3). After irrigation and recovery of water status, tree water isotopic composition became more depleted during midday compared to morning and evening. This likely occurred because transpiration is at its peak at midday (Figure 7a), and trees are likely to withdraw more water from the soil. Thus, xylem and phloem water isotopic composition after the irrigation and recovery in water status probably reflected the more depleted irrigation water (Figure 7a), especially at midday.

Additionally, our subdaily isotopic measurements support the variable radial transport pathway between xylem and phloem proposed by Steppe et al. (2012). Phloem and xylem isotopic composition were more similar during predawn and morning times and more distinct during phloem water refilling and midday prior to irrigation events. During predawn periods, flow of water from phloem to xylem—the so-called Münch's counterflow (Münch, 1930; Thompson & Holbrook, 2003) under low water potential, resulted in an isotopic exchange with xylem water, decreasing the isotopic difference between pools. Previous studies have shown that flow from phloem to xylem is lower during the day than it is at night (De Schepper & Steppe, 2010; Windt et al., 2006), and this may explain our observed isotopic similarity during predawn.

### 4.3 | Implications for perceptual models of tree water use

Early evidence of more enrichment of phloem water in relation to xylem (e.g., Adar et al., 1995; Cernusak et al., 2005) perhaps led to the exclusion of phloem (i.e., inner bark) during tree water source investigations. However, the removal of phloem tissue at the time of sampling does not exclude its effect on xylem water, since xylem and phloem are functionally and hydraulically interconnected (Pfautsch, Hölttä, & Mencuccini, 2015). Thus, understanding the isotopic composition of phloem water might be important given that such hydraulic coupling exists, and evidence of isotopic difference between these pools, along with potential increase in $\Delta_{phloem-xylem}$ during periods of tree water stress.

Isotope fractionation between xylem and phloem water is likely influenced by the size of phloem storage that needs to be refilled in relation to total transpiration rates. Despite the small observed daily contribution of phloem water storage in relation to daily transpiration, we did observe a small but statistically significant difference between these pools within the tree. Although significant, the observed differences did not affect overall results in tree water source partitioning when including phloem water along with xylem water in assessment reported by previous study conducted in this lysimeter (Nehemy et al., 2021). However, if our phloem water storages were larger, and there were days when xylem would need to recharge a more

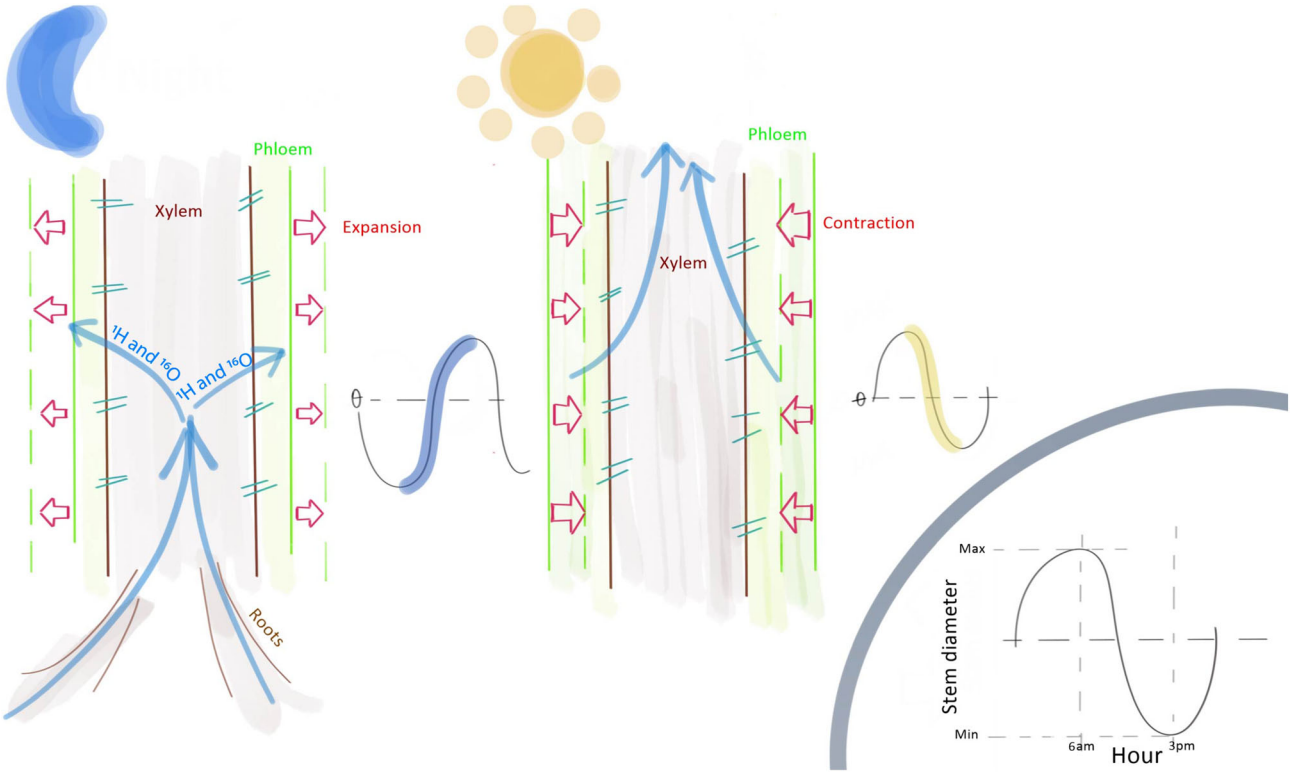


**FIGURE 8** Perceptual model showing water movement between phloem and xylem throughout the day along with diameter variation (i.e., variation in volume of elastic storage). During phloem water refilling (observed between 15:00—early in the evening, after minimum stem shrinkage, and 2:00—before maximum stem expansion), lighter isotopes are preferentially transported into the phloem when water transport is modulated by aquaporins, and xylem can become systematically enriched over time. The transport via aquaporins is also enhanced during periods of water stress, which may result in more differences in isotopic composition between phloem and xylem

considerable volume of phloem water, equivalent to, for example, the 15% of daily transpiration as observed in other studies for larger trees (see Table 3 in Betsch et al., 2011), we speculate that xylem would likely be much more enriched than original soil water sources as a result of preferential recharge of lighter isotopes into the phloem (Figure 8). Here, the small phloem contribution (<1%) already resulted in detectable and measurable differences. We, thus, propose that the enrichment effect of phloem recharge in xylem water is more likely to be observed in species with characteristically high proportional use of internally stored phloem water in relation to daily transpiration, along with lower xylem water capacitance. Xylem water capacitance is influenced by wood anatomy and shows an inverse relationship to wood density (Jupa et al., 2016; Oliva Carrasco et al., 2015). Treydte et al. (2021) showed recently that the radial water transport and water exchange between sapwood and phloem are also influenced by wood density. Although our measurements are within reported values in the literature for phloem storage in relation to transpiration fluxes (see Table 3 in Betsch et al., 2011), further work should also investigate the effect of the reversible expansion of sapwood along with phloem (e.g., Steppe et al., 2015) for a more accurate representation of phloem water storage.

A recent study has proposed that cryogenic extraction biases determination of xylem water isotopic composition (Chen et al., 2020). They suggest that cryogenic extraction results in a significant depletion in xylem $\delta^2H$ and that this depletion is directly linked to variation in sample relative water content. Chen et al. (2020) suggested that low water content in an extracted sample can result in higher isotopic depletion of xylem water during cryogenic extraction. This interpretation has been challenged by Evaristo et al. (2021) and Y. Zhao (2021). Our measured phloem and xylem relative water content (Figure S3) showed greater water content in phloem in relation to xylem samples (on average 16% greater). Higher water content in phloem is expected given tissue characteristics and has been previously reported (Treydte et al., 2021). Also, the absolute amount of water extracted per vial (xylem or phloem) was always greater than 0.72 ml. Based on Chen et al. (2020) observations, if the isotopic difference between phloem and xylem water was an artefact of the extraction system, one would expect more depleted isotopic values in xylem water (given its lower water content in relation to phloem). However, we observed the opposite. Further, $\Delta_{phloem-xylem}$ was observed in both $\delta^2H$ and $\delta^{18}O$ and not only in $\delta^2H$ as suggested by Chen et al. (2020). Thus, in our study, we can very likely refute that the resulting difference between phloem and xylem water is an artefact of cryogenic extraction. Additionally, a previous greenhouse experiment that also investigated the cryogenic extraction impact on xylem water using *S. viminalis* cuttings (the same species used in this experiment) showed no bias for $\delta^2H$ and only a small but consistent enrichment in $\delta^{18}O$ above soil water (Newberry et al., 2017). In situ monitoring of xylem (e.g., Landgraf et al., 2021; Marshall et al., 2020; Volkmann, Kühnhammer, et al., 2016) and phloem water isotopic composition could likely improve uncertainties related to extraction method and would provide higher temporal-resolution measurements. However, in situ monitoring of phloem (i.e., inner bark) has not been tested yet. This approach could also address potential issues related to high-frequency invasive xylem and phloem sampling.

Additional investigations should test whether small xylem water offsets in relation to LMWL and sources can be influenced by phloem water storages and the exchange between xylem and phloem water. Such investigations are important given growing evidence of phloem depleted isotopic composition (Treydte et al., 2021; Wang et al., 2021). Our results and recent discussion in the literature highlight that monitoring phloem water isotopic composition (i.e., inner bark) is important to improve our understanding about mechanisms dominating radial tree water transport (Gessler, 2021). The impact of phloem water in xylem isotopic composition could be potentially larger in trees with larger internal phloem water storage pools. Of course, soil water isotope fractionation would be also reflected in the xylem when trees rely on those sources. Phloem water could further compliment discussions regarding heterogeneity in isotopic composition within plant water tissue (Barbeta et al., 2022). However, the uncertainty resulting from stable isotope investigation of tree water source could be reduced by considering tree hydraulic measurements and phloem water sampling as a complement to xylem water tracing (Nehemy et al., 2021; Treydte et al., 2021).

## 5 | CONCLUSION

We found that, on average, xylem water is more enriched than phloem water. Phloem and xylem water were highly correlated supporting the tight hydraulic connectivity between these transport pathways within the trees. Phloem and xylem $\delta^2H$ and $\delta^{18}O$ differences were related to daily use of storage and transpiration rates. We propose that the observed phloem water depletion results from radial transport between phloem and xylem. However, these findings contrast with early findings and general expectations that phloem water should have heavier isotope ratios when compared with xylem and reflect evaporative enrichment in leaves. Our observations appear to show that the replenishment of phloem water is associated with preferential transport of lighter isotopes during radial water transport, which could be attributable to water flow mediated by aquaporins. However, more studies are needed to understand isotope fractionation associated with xylem–phloem exchange. Observations of phloem water isotope compositions are important to improve our understanding about plant hydraulic and hydrological processes. Additionally, our findings prompt us to suggest that improved understanding of phloem isotopic composition and its influences on xylem water represents an important avenue towards generally better understanding and interpreting stable isotopes values in trees.

### ACKNOWLEDGEMENTS

We thank Kim Janzen for assistance with laser and mass spec analyses. We thank Dyan Pratt, Mitra Asadollahi and the Laboratory of Ecohydrology at EPFL (ECHO/IIE/ENAC/EPFL) for assistance throughout the experiment. We thank Pedro Hervé-Fernández for useful discussions along the way and Clément Duvert and an

anonymous reviewer for constructive and useful feedback that improved the final version of this paper. This research was supported by the American Geophysical Union (AGU) —Horton Research Grant 2019 awarded to Magali F. Nehemy, an SNSF Ambizione Grant (No. 179978) to Marco M. Lehmann and an NSERC CREATE in Water Security Grant and an NSERC Discovery Grant to Jeffrey J. McDonnell.

## DATA AVAILABILITY STATEMENT

Data presented in this paper are available at https://doi.org/10.5281/zenodo.4037240 (Nehemy et al., 2020), under Creative Commons Attribution (CC BY) licence.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

**How to cite this article:** Nehemy, M. F., Benettin, P., Allen, S. T., Steppe, K., Rinaldo, A., Lehmann, M. M., & McDonnell, J. J. (2022). Phloem water isotopically different to xylem water: Potential causes and implications for ecohydrological tracing. *Ecohydrology*, e2417. https://doi.org/10.1002/eco.2417