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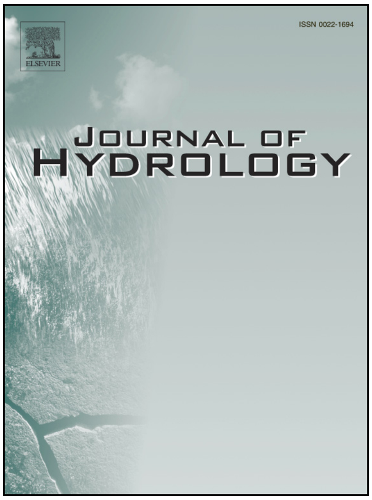

Research papers

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Examination of the ecohydrological separation hypothesis in a humid subtropical area: comparison of three methods

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Abstract: The ecohydrological separation between soil water sources for plant water uptake and groundwater recharge has been recently examined in various climate zones primarily based on isotopic composition of water. The existence of the ecohydrological separation has profound implications for mechanistic ecohydrological modeling and water resource management. However, it is still unclear when and where the ecohydrological separation occurs, especially in humid regions. In this study, high frequency sampling of precipitation, bulk soil water, groundwater and twig xylem water for hydrogen and oxygen isotope composition measurement was conducted in a humid subtropical site in the central southern China from March 2017 to April 2018. We examined evidence of the ecohydrological separation with three methods (dual-isotope space, line-conditioned excess (lc-excess), and the piecewise isotope balance (PIB) method). The results show that the isotope compositions of plant xylem and bulk soil water are not distinguishable from those of precipitation water on the dual-isotope space due to a weak evaporation effect at the study site, indicating that there is no evidence of the ecohydrological separation. However, the other two methods support the ecohydrological separation in this humid area, with the results from the PIB method revealing more temporal details. The present study suggests that the ecohydrological separation can happen in subtropical humid climate. It is more likely to occur in spring and winter at the study site when plant-accessible water pool has been replenished by antecedent precipitation, while ecohydrological connection seems to occur during winter snowmelt. With the limitations of three methods, the caution should be taken when only one method is

applied in examining the ecohydrological separation in such an environment.

Keywords: ecohydrological separation; stable isotopes; plant accessible water; water replenishment; lc-excess; piecewise isotope balance method

1 Introduction

Soil water is the immediate water source for plants in the terrestrial ecosystems, thus, the plant-accessible water in soil is essential for plant growth. Generally, soil water has been assumed to be a well-mixed water pool (Hewlett and Hibbert, 1966; McCutcheon et al., 2017), which means that plants extract water from the same pool that drains into groundwater and/or flows into streams. However, recent studies suggest that streams and plants appear to have water originated from two different water pools: mobile water for groundwater/stream recharge, and immobile water for plant use. Such a phenomenon was first proposed in Brooks et al. (2010) based on distinguished isotopic composition between stream and plant water in a Mediterranean climate zone. Thereafter, this phenomenon was referred to as the two water worlds (McDonnell, 2014) or the ecohydrological separation hypothesis (Geris et al., 2015; McCutcheon et al., 2017).

Evidence for the ecohydrological separation hypothesis has been reported in various climate zones (Bowling et al., 2017; Brooks et al., 2010; Evaristo et al., 2016; Gierke et al., 2016; Goldsmith et al., 2012; Hervé-Fernández et al., 2016; McCutcheon et al., 2017; Zhao et al., 2018). Meanwhile, at the global scale, Evaristo et al. (2015) suggest that the ecohydrological separation occurs at 80 % of the sites, and Good et al. (2015) report that 38 % of surface water comes from the plant accessible soil water pool. These studies suggest that the ecohydrological separation may commonly occur. These findings not only challenge the assumptions (e.g. water in soils is well-mixed) that have been commonly accepted in conceptualizations of hydrological and biogeochemical cycles, but also have practical implications for agricultural fertilizer application, surface-induced groundwater pollution, and fate and transport of subsurface contaminants (Phillips, 2010).

The reported degrees of the ecohydrological separation vary greatly among different studies. The highest separation was estimated by Brooks et al. (2010) in a Mediterranean climate, in which the separation exists from the rainy season (winter) to the next wet-up period in autumn. However, most other studies show that the ecohydrological separation seems to be temporal and more likely to occur during dry periods (Brooks et al., 2010). In humid climate, the reported results of ecohydrological separation are not consistent among studies. For example, no marked separation was found in wet environment (Geris et al., 2015; Hervé-Fernández et al. 2016). Two studies (Qian et al., 2017; Zhao et al., 2018) with investigations being conducted in the same climate zone (subtropical monsoon climate) showed contrasting results of the ecohydrological separation. The inconsistency in these previous studies suggests it is still unclear when and where the ecohydrological separation occurs, especially in humid regions.

Despite increasing evidence of the ecohydrological separation, several potential problems arise from current methods in the investigation of the ecohydrological separation. Firstly, current methods for assessing the ecohydrological separation do not necessarily work for humid environments. The commonly used methods for assessing the ecohydrological separation hypothesis are the so called dual-isotope space and line-conditioned excess (lc-excess) assessment. For the dual-isotope space, groundwater and stream water generally plot on or near a local meteoric water line (LMWL) based on the seasonal δ^2H and δ^{18}O variations in precipitation. Soil water usually varies along a soil water evaporation line (SWEL) with different degree of deviation from the LMWL due to different evaporation and mixing processes. The ecohydrological separation is considered occurring when plant water is plotted to the right of the LMWL and close to the SWEL, with isotopic distinction from mobile water (stream and ground water). For the lc-excess assessment, this variable is to quantify the isotopic distance of a water sample from the LMWL (Landwehr and Coplen, 2006). Theoretically, precipitation lc-excess values will fluctuate near zero, so do the lc-excess values of mobile water. Soil water that has undergone evaporation will have a negative lc-excess value. Thus, evaluation of the ecohydrological separation is based on the significant difference in the lc-excess values between plant water and mobile water. Note that these two methods are effective based on the assumption that plants use water has undergone notable evaporation. However, this assumption may be problematic for an environment where evaporative effects on the isotopic composition of environmental waters are not significant. This may be one reason for the inconsistent results of the reported ecohydrological separation in humid climate as we mentioned above.

Secondly, common soil water samplings do not necessarily reflect the isotopic composition of water used by plants. Recently, Sprenger et al. (2015) gave an overview of various methods of soil water extraction for stable isotope analysis, which shows that different methods extract pore waters of different mobility. For those previous studies on the ecohydrological separation, bulk soil water that includes both mobile and immobile (matric-bound) water (Brooks et al., 2010) was sampled with the cryogenic vacuum distillation. In addition to bulk soil water sampling, some studies also extracted mobile soil water using suction lysimeters (Brooks et al., 2010; Hervé-Fernández et al., 2016), centrifugation (Geris et al., 2015), or wick samplers (Gierke et al., 2016). As most studies on the ecohydrological separation assume that plants rely on the immobile soil water, it should be reasonable to investigate isotope composition of immobile water to reflect the isotopic composition of water used by plants. However, it is difficult to sample the immobile soil water that is really extracted by plants based on current technologies. Although the isotopic composition of bulk soil water (generally based on point measurements) can reflect that of the water acquired by plants in some case (e.g. during dry periods), such bulk soil water samplings may give uncertainties in inferring the isotopic composition of water acquired by plants during wet periods.

Finally, the different degrees of the ecohydrological separation reported in the literature can be partly resulted from a low frequency of isotope sampling. As Hervé-Fernández et al. (2016) mentioned that sampling frequency is still one of the most significant limitations in ecohydrological studies, and many field campaigns in previous studies only have samples collected a few times per season (e.g. wet-dry season) (Brooks et al., 2010; Evaristo et al., 2016; Geris et al., 2015; Goldsmith et al., 2012). A low sampling frequency likely misses the detail of temporal variation. Such temporal variations likely provide important information to understand the mechanism of ecohydrological separation. High frequency sampling of plant water and soil water for isotopic composition analysis is thus especially beneficial for improving understanding of the ecohydrological separation (McDonnell, 2014).

In this study, we examined evidence of the ecohydrological separation in a humid subtropical area based on high frequency sampling (two to four times in a month). In addition to these two commonly used methods mentioned above, we adopted a new approach, referred to as the piecewise isotope balance method (see section 2.6 for details). The method requires high frequent samplings of isotopic composition of plant xylem water and precipitation. Isotopic composition of plant xylem water generally reflects the integrated isotopic compositions of plant-accessible soil water with the assumption that no isotope fractionation occurs during root water uptake and water flow in plant xylem (Flanagan and Ehleringer, 1991; White et al., 1985). With this assumption, it is possible to use plant water isotopes for investigating plant-accessible water replenishment, which can be used to infer the ecohydrological separation possibility.

In this study, we aimed to answer the following questions: (1) To what extent does ecohydrological separation happen in a humid subtropical area? (2) What is the difference in assessing the ecohydrological separation hypothesis in this humid subtropical area by using the three methods (dual-isotope space, lc-excess assessment and the piecewise isotope balance method)?

2 Materials and methods

2.1 Study site

The field experiment was conducted in a grove, located in the suburb of Changsha city in the central southern China (28°22′8.02″N, 112°45′42.91″E, 51 m a.s.l.) (Fig. 1). This area has a humid subtropical monsoon climate, generally with a dry and cold winter, and a wet and hot summer. Mean annual air temperature is about 17.4°C, and mean annual precipitation is 1447 mm (mainly distributes from March to June). Resulting from different air mass influences in the summer monsoon season, precipitation distributes unevenly between months, often leading to low precipitation in midsummer (Luo et al., 2016). Soils at this site are silty loam, with clay, silt and sand content about 20.1%, 75.7% and 4.2%, respectively. The average soil bulk density at this site is about 1.32 g cm^{-3}. The soil thickness at the study site exceeds 4 m based on a soil profile near the study grove (see the Fig. S1 in the supplementary material).

[insert Fig.1 about here]

The study grove is dominant by evergreen broadleaf trees *Cinnamomum camphora*, mixed with some *Cunninghamia Lanceolata*, *Pinus massoniana*, and sparse grasses on soil surface. We chose *C. camphora* as our sample trees. The average age and height of the trees are about 13 years and 9 m, respectively. Most roots (85%) of *C. camphora* generally distribute in the shallow soil layer (0-40 cm) in the study area according to Yao et al. (2003). We selected a plot of 20×40 m^2 as the target site. This site is flat (slope <3°), and thus the effect of lateral flow on root zone water can be neglected.

2.2 Sampling

Soil and twig xylem water sampling was conducted from March 2017 to April 2018, with a high sampling frequency (two to four times in a month). For twig samples, only non-photosynthetic stem sections (about 4 cm in length and 1.5-2 cm in diameter) were collected from 3-6 individual trees. The bark was quickly removed before each sample was immediately sealed into glass bottles with parafilm. Soil samples were collected every 10 cm to a depth of 130 cm using a soil corer driven by an engine harmer (Christie Engineering Pty Ltd., Australia). Three replicates of soil samples were collected near the trees from which the twig samples were taken. Meanwhile, for each 10-cm soil section, a 2-cm soil core (3.8 cm in diameter) was collected for soil water content and bulk density analysis. On the same day, groundwater was collected from a well, which was about 30 m away from the sampling site. This well has a depth of 20 m with approximately 18 m to the water table. It is pumped daily for the owners' family use. Water from this well very likely represents the groundwater in this study area (reflected by the elevation in Fig. 1). The well was perched by 30 minutes pumping at a rate about 1 m^3/h before water samples were collected. All samples were sealed in airtight vials, placed in a cooler box, and transported to the lab and stored in a refrigerator before analysis.

Rainfall water samples were collected at an automatic meteorological station located in Hunan Normal University, which is about 26 km away from the study site.

Continuous precipitation sampling (based on precipitation event (>0.1 mm)) for isotope analysis has been conducted for more than 8 years at the site. We use these high-frequency data set to represent the isotopic composition of precipitation at the study site with an assumption that there is no significant difference between these two sites. This is supported by high similarity in the precipitation event size pattern and isotopic composition between these two sites, as shown in Fig. S2 in the supplementary material. Precipitation sampling includes rainfall water and snowfall (if any). More information of sampling at this site can be found in (Li et al., 2015; Wu et al., 2015).

2.3 Soil water content

Soil volumetric water content (θ, cm^3/cm^3) was automatically monitored using a frequency domain reflectometry (FDR) system ET100 (Insentek Co., Ltd, China). This equipment estimates volumetric water content based on measurements of the apparent dielectric constant in the soil. We installed ET100 (6.3 cm in diameter) to a depth of 100 cm nearby the sample trees. Each ET100 has two sensors at each layer with 10 cm intervals for monitoring soil water content and temperature (average of each 10 cm soil layer). The sampling interval was set as one hour.

2.4 Micrometeorological data

An automatic weather station (WeatherHawk-232, USA) was installed in an open area approximately 50 m from the grove. Meteorological variables, including precipitation, air temperature, solar radiation, relative humidity and wind speed were measured at 30 min intervals.

2.5 Data analysis

Soil and twig xylem water samples were extracted using an automatic water extraction system LI-2100 (LICA United Technology Limited, China) based on the cryogenic vacuum distillation method (Li et al., 2015; Wu et al., 2015). Generally, a complete water extraction should be processed to obtain unfractionated water samples. In this study, we set moisture extraction time of 150 and 180 min for soil and twig samples, respectively, in order to collect more than 99% of the water from these samples. Isotopic composition (δ^2H and δ^{18}O) of the extracted water, precipitation and well water were analyzed with a Cavity Ring-Down Spectroscopy (CRDS) Isotopic Water Analyzer (Los Gatos Research, Inc., Mountain View, USA) at the Key Laboratory of Resources and Environmental Sciences, Hunan Normal University. δ^2H and δ^{18}O values are generally expressed in delta notation (δ) as

$$\delta = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1\right) \times 1000 \tag{1}$$

where δ (‰) is the ^{2}H/H and ^{18}O/^{16}O ratio relative to Vienna Standard Mean Ocean Water (V-SMOW), R is the ratio (^{2}H/H and ^{18}O/^{16}O) of sample and standard (V-SMOW), respectively. Accuracy of the IRIS analyzer was ±0.2 ‰ for δ^{18}O and ±0.6 ‰ for δ^2H.

Previous studies have shown that organic materials in the extracted plant and soil water samples may cause spectral interference and lead to incorrect isotope values in the IRIS analyses (Schultz et al., 2011; West et al., 2010; Zhang et al., 2017). However, such problems can be corrected by a standard curve method (Schultz et al., 2011). Methanol and ethanol are two main organic contaminations in the extracted water samples. Firstly, we prepared water samples of known concentrations of methanol and ethanol, and obtained the correcting curve according to Wu et al. (2014). Then, the Spectral Contamination Identifier (LWIA-SCI) post-processing software was used to identify and quantify spectral contamination. Only methanol contamination was found in twig xylem water samples in this study. Therefore, all isotope values for the extracted twig xylem water were corrected for the methanol contamination.

2.6 Methods for the ecohydrological separation assessment

Three methods, dual-isotope space, lc-excess, and the piecewise isotope balance (PIB) method, were applied to examine the ecohydrological separation in this study. For the dual-isotope space method, the ecohydrological separation of soil water sources for trees and groundwater is determined by the isotopic distinction between plant xylem water and groundwater with different degrees of deviation from the LMWL. Note that the LMWL in this study was produced based on isotopic composition of daily precipitation with the amount larger than 2 mm, because we found there was no contribution of small rainfall events (<2 mm) to soil water.

For the lc-excess assessment, the isotopic composition of all water samples was compared to the LMWL by using lc-excess, which quantifies δ^2H deviations from the LMWL. In this study, lc-excess (‰) was calculated following Landwehr and Coplen (2006):

$$\mathrm{lc-excess} = \delta^2\mathrm{H} - a \times \delta^{18}\mathrm{O} - b \tag{2}$$

where a and b are the slope and intercept of the LMWL. Generally, the lc-excess indicates the degree of offset of the sampled waters from the presumed source (e.g. the LMWL). Once the lc-excess values of plant xylem water are negative and significantly different from those of groundwater, the ecohydrological separation is considered occurring.

For the piecewise isotope balance (PIB) method, based on high frequent sampling of twig xylem water and precipitation, we calculated the proportion of plant-accessible water (reflected by the isotopic composition of xylem water) within the root zone replenished by precipitation between two consecutive sampling events. Here we assumed that precipitation is the main source for plant-accessible water replenishment. Meanwhile, evaporation effects on water sources for plant use were neglected due to weak evaporation influencing the isotopic composition of waters at the study site. In this study, we only used δ^{18}O values (no fractionation of oxygen isotope was found during root water uptake in the literature) for quantifying the replenishment proportion (β, %), using the following equation:

$$\beta = \left(\delta_{x,i} - \delta_{x,i-1}\right) / \left(\delta_{\sum p} - \delta_{x,i-1}\right) \times 100 \tag{3}$$

where $\delta_{x,i}$ and $\delta_{x,i-1}$ are δ^{18}O values of twig xylem water between two consecutive sampling events. $\delta_{\Sigma p}$ represents the volume-weighted mean precipitation δ^{18}O value between two consecutive samplings. Note that β was assumed to be 0 when $\delta_{x,i} < \delta_{x,i-1}$, and $\delta_{\Sigma p} > \delta_{x,i-1}$ or when $\delta_{x,i} > \delta_{x,i-1}$, and $\delta_{\Sigma p} < \delta_{x,i-1}$. In addition, if the difference between $\delta_{x,i}$ and $\delta_{x,i-1}$ was very small (smaller than the accuracy (0.2 ‰) for δ^{18}O) during a short period when θ was high and did not change much between two sampling intervals, β was assumed to be 0. Only two such cases were found during the sampling period between 26 March and 9 April, and between 29 April and 11 May 2017.

2.7 Statistical analyses

Statistical analyses were conducted using Origin 9.0 software (OriginLab, USA). The Kruskall-Wallis test (ANOVA) was used to analyze differences in isotope composition and lc-excess values among different water pools. Comparison of isotope composition between different periods was analyzed via the non-parametric Mann-Whitney test.

3 Results

3.1 Seasonal variations in isotopic composition for specific waters

Fig. 2 shows the range of isotopic composition for different water pools. A large variability for H and O isotope composition is shown in precipitation with low values in summer and high values in spring, which forms a local meteoric water line (LMWL) as $\delta^2H=8.69\times\delta^{18}O+19.99$ ($R^2=0.98$). The volume-weighted mean precipitation values for $\delta^{18}O$ and δ^2H are -7.09 ‰ and -42.56 ‰, respectively. Groundwater, bulk soil water and twig xylem water isotopes plot close to or along the LMWL, indicating that the evaporative effect is not significant in these water samples. There are no significant differences in mean $\delta^{18}O$ of twig xylem water, groundwater and precipitation, but mean $\delta^{18}O$ of soil water is significant different ($P<0.05$) with them. Such difference pattern for $\delta^{18}O$ is not consistent with that for δ^2H. For groundwater, $\delta^{18}O$ and δ^2H give a small seasonal variation, in a range from -6.7 to -5.4 ‰ for $\delta^{18}O$ and from -39.2 to -32.8 ‰ for δ^2H (Fig. 2).

Bulk soil water shows a notable temporal and spatial (vertical) variation in isotope values during the study period (Fig. 2 and 3b). Soil water are more enriched with heavy oxygen isotope in spring and more depleted in summer and autumn, reflecting seasonal variation of precipitation inputs. The mixing processes of $\delta^{18}O$ within the soil profile over time are illustrated in Fig. 3b. The $\delta^{18}O$ of bulk soil water decreases with depth between March and early June 2017. However, the largest decrease of $\delta^{18}O$ in shallow soil water (0-40cm) occurs in mid-June due to more depleted oxygen isotope inputs from precipitation (Fig. 3c). The low $\delta^{18}O$ in shallow soil persists to the end of autumn, $\delta^{18}O$ in shallow soil increases in the following season due to the replenishment and mixing by precipitation. $\delta^{18}O$ of deep soil water (>60cm) maintains relatively stable between mid-summer and winter, the corresponding θ does not change much over time either.

Twig xylem water $\delta^{18}O$ has a small seasonal variability in comparison with that of precipitation (Fig. 2a). $\delta^{18}O$ keeps relatively constant (-3.89 ± 0.33 ‰) in spring, and decreases significantly ($P<0.05$) in June and to a low value of -7.26 ± 0.18 ‰ on 14th July. This decrease indicates that root-accessible water has been replenished. Twig water $\delta^{18}O$ has been relatively stable (-7.19±0.29 ‰) from July to February except for a slightly increase during a short dry period (21 rain-free days) in late July, and two decreases in mid-August and late October respectively due to replenishment by the precipitation inputs. The $\delta^{18}O$ value of twig xylem water is very close to that of snowfall and the groundwater in the early February, indicating a relatively full mixing in the soil and ecohydrological connection during the winter snowmelt. Twig xylem water $\delta^{18}O$ shows a step change during the following spring 2018. In addition, $\delta^{18}O$ of twig water matches that of shallow soil water (0-40 cm) for most of the time during the study period (Fig. 3a and 3b).

[insert Fig. 2 about here]

[insert Fig. 3 about here]

3.2 Examination of the ecohydrological separation

Isotopic values of all water samples are close to the LMWL as shown in the dual-isotope plots in Fig. 2, which indicates that these waters have not experienced significant evaporation. Meanwhile, twig xylem water and groundwater are not isotopically distinct (reflected by $\delta^{18}O$). Thus, this result does not support the ecohydrological separation based on the dual-isotope method that has commonly been used in other studies.

Precipitation shows both positive and negative lc-excess values, but they are not significantly different ($P<0.05$) from 0. The lc-excess of groundwater is significantly less than 0 ($P<0.05$) (Fig. 4). The lc-excess values of soil water differ significantly from 0. Twig xylem water has the most negative lc-excess values, and is significant different ($P<0.05$) from that of groundwater. This suggests that water extracted by plants (twig xylem water) is isotopically distinct from that recharges groundwater. Thus, this result supports the ecohydrological separation hypothesis in this study.

Although the result based on the lc-excess method suggests that ecohydrological separation occurs in this subtropical region, it does not show the temporal variation in ecohydrological separation and its duration. The lc-excess values of twig xylem water are consistently different from that of groundwater during the whole study period (Fig. 4), which seems to suggest that ecohydrological separation happens all the time at this site. However, this is unlikely the case as there have not been similar results reported in the literature.

Therefore, in order to investigate when the ecohydrological separation happens at this site and how long it lasts, we investigate the dynamics of twig xylem water isotope composition to examine the ecohydrological separation. The δ^{18}O values of twig water show relatively constant values between March and May 2017 while those of precipitation fluctuate. This result indicates that precipitation is likely to have bypassed the root-accessible water pool, suggesting the ecohydrological separation occurs. Such an ecohydrological separation seems to happen in other periods at the study site (e.g., late November to January, and April 2018) based on the relatively constant isotope signature of twig xylem water. The ecohydrological separation is further examined by the PIB method, which calculates plant-accessible water replenishment, shown in the following section.

[insert Fig. 4 about here]

3.3 Plant-accessible water replenishment

Fig. 5 shows the percentage of precipitation contributes to plant-accessible water based on δ^{18}O of twig water and precipitation. The results indicate that no replenishment occurs between March and early May 2017. This means that plant-accessible water does not mix with precipitation during this period, and that the plant-accessible water pool replenished by antecedent precipitation has maintained more than two months. Some similarities in the replenishment pattern can also be found in spring 2018. These replenishment patterns support the ecohydrological separation analyzed above. The first large replenishment (41 %) occurs in later May, and about 32 % replenishment happen again between 7th June and 9th July 2017 due to heavy precipitation (making θ_a to field capacity). During the dry period (from July to October 2017), plant-accessible water has been replenished for several times. Such replenishments are easy to occur when θ is significantly low especially in the topsoil (Fig. 3c). However, the isotopic composition of twig xylem water in September is close to that in August (Fig. 3a). This short life of replenishment signal suggests that they may occur only in the shallow root zone, thus maintain for root water uptake only for about 2~3 weeks (reflected by the variations in δ^{18}O values of twig water in Fig. 3a). In addition, significant low replenishment (0~5.8%) is inferred between December and mid-January, suggesting an ecohydrological separation during this period. The highest replenishment (54.5 %) occurs between the later January and early February, which is mainly due to the contribution of snowmelt (snowfall accounts for about 50 % of precipitation during this period). The δ^{18}O value of twig xylem water observed in the early February is close to that of the snowfall (Fig. 3a) and groundwater, indicating the possibility of ecohydrological connection. Such isotope signature of the replenished plant-accessible water maintains until the later March before the next replenishment event (reflected by the δ^{18}O values of twig xylem water in Fig. 3a, and Fig. 5).

[insert Fig. 5 about here]

4 Discussion

4.1 Evidence of the ecohydrological separation in subtropical humid climate

The ecohydrological separation is observed in this subtropical humid study site. The separation is more likely to occur in spring and winter season based on the integrated results from the lc-excess and PIB methods (Fig. 4 and 5). Such a pattern of separation differs from two other studies in the subtropical monsoon climate zones in China (Fig. 6). Qian et al. (2017) suggest no ecohydrolgical separation in a subtropical riparian area, based on the dual-isotope space and lc-excess method. This may be due to significant contribution of river water and groundwater to plant water uptake in their riparian zone. Zhao et al. (2018) report the existence of ecohydrolgical separation in May, Jun, October and November, but not during wet season in their study site (between July and September) based on the lc-excess method. Nevertheless, Evaristo et al. (2016) report that occurrence of ecohydrological separation in wet season for a humid tropical region. The different results of these studies suggest complexity of the ecohydrological separation in humid climates. Based on the results from this present study and previous studies, the ecohydrological separation very likely occurs in tropical and subtropical humid climates, but with temporal pattern less predictable.

[insert Fig. 6 about here]

In this study, the ecohydrological separation is more likely to occur in spring and winter, and can last for a few months according to the PIB-inferred replenishment events. Generally, small pores are filled first and dried out last, while large pores will be a dominant pathway of infiltration water moving through the soil profile once small pores are fully filled (Brooks et al., 2010). Water in small pores is relatively immobile, and has the longest residence time (Brooks et al., 2010). Therefore, the common possibility for the separation in spring and winter at the study site is that immobile water (plant-accessible water) for tree transpiration has been replenished by initial precipitation during the wet-up period prior to these two seasons (such as high replenishment in November 2017 or the early spring 2018 in Fig. 5), and such immobile water is sufficient for root water uptake. But the condition for the separation seems to be different between spring and winter. For spring, the separation occurs under the condition of high soil water content and atmospheric demand, precipitation over the remainder of the spring bypasses root zone through larger pores and preferential flow paths, and does not seem to mix fully with immobile soil water used by plants (reflected by relatively constant values of xylem water δ^{18}O in Fig. 3a). For winter, the pre-existing immobile water probably persists in small pores for a few months due to low depletion from evaporation and tree transpiration during this period (based on sapflow measurements, data not shown), and thus does not mix with the subsequent precipitation inputs. On the other hand, the ecohydrological connection occurs during snowmelt in late winter at the site, indicating that snowfall events (occurred frequently at the study site based on records at the Changsha weather station) are likely to lead to the end of separation and be an important water source for root zone water replenishment.

4.2 Comparison of three methods for estimating the ecohydrological separation

The results of the ecohydrological separation among these three methods are not consistent. The dual-isotope space method does not show evidence of the ecohydrological separation, while the other two support ecohydrological separation at the study site. Due to weak evaporation isotope effect at the study site, the isotope compositions of plant xylem and soil water are not distinguishable from precipitation water, leaving the dual-isotope method without a basis. Even though significant deviation of plant and soil water from the LMWL, the dual-isotope method cannot give an unambiguous determination of water source(s) of plant use (Bowling et al., 2017).

The lc-excess assessment gives clear evidence of ecohydrological separation based on the significant difference in lc-excess values between twig xylem water and groundwater. Few previous studies have focused on temporal variations in lc-excess values of different water pools due to low frequency of isotope sampling. Hervé-Fernández et al. (2016) and Zhao et al. (2018) suggest that the ecohydrological separation does not occur during the wet season based on temporal variations in lc-excess values. However, temporal variations in lc-excess values in this study infer a high degree of separation during the study period. The consistently deviation of the lc-excess values between twig xylem water and groundwater should not be caused by evaporation at the study site. The potential cause for this is that the pre-existing plant-accessible water cannot be completely replaced by infiltration precipitation water (also confirmed by the plant-accessible water replenishment proportion (< 60 %) in Fig. 5). Therefore, the lc-excess assessment does not seem to be a reliable method, and its results need a further confirmation with other additional evidences for assessing the ecohydrological separation.

The PIB method shows more details of the ecohydrological separation based on plant-accessible water replenishment in this humid subtropical area. The pattern of replenishment proportion is similar with the variation of δ^{18}O values of xylem water in response to precipitation (Fig. 3a and 5). The advantage of this method is that it shows when and how long the ecohydrological separation occurs and lasts. Furthermore, the PIB method only uses isotope data of plant xylem water and precipitation. It does not rely on isotopically distinction between plant xylem water (immobile water) and ground/stream water (mobile water) as such distinctions may not relate to soil water mobility (McCutcheon et al., 2017).

Despite the obvious advantages of the PIB method, some limitations still need to be noted. The first limitation is that we neglect the evaporation effects between each sampling interval when calculating replenishment proportion. Nevertheless, the effects of evaporation on isotope are not significant in this study, which provides a good experiment condition to apply the PIB method. The second limitation is that there exist some uncertainties in estimation of replenishment proportion based on mean isotopic values of precipitation inputs between two consecutive sampling events. It is difficult to accurately estimate the relative contribution of multiple precipitation events in any a sampling interval. In this study, a volume weighted isotope composition was applied, which can certainly lead to an error in PIB calculation. With an understanding that small events do not contribute to root zone moisture replenishment, precipitation events smaller than 2 mm were excluded. This is the best approximation we can have to apply for the PIB method at this stage. The third limitation is that the PIB-inferred replenishment does not necessarily suggest ecohydrological connectivity, which needs to be confirmed with other evidences. In addition, the lateral flow should be considered if the study is conducted at a sloped site. Future studies need to take consideration of evaporation effects as well as lateral flow into investigations to further test the PIB method. In short, there are no perfect methods for assessing the ecohydrological separation among current studies, and caution should be taken in choosing a method for examining the ecohydrological separation in such a humid environment.

5 Conclusions

The ecohydrological separation hypothesis was assessed based on high frequency of isotope sampling in the humid subtropical region of the central southern China. In this study, bulk soil water, groundwater and twig xylem water isotopes plot close or along the local meteoric water line (LMWL), with isotopically similarity between groundwater and twig xylem water. This indicates the evaporation effect on isotopes of these waters is not significant. Evaluations of the ecohydrological separation among these three methods are not consistent. The dual-isotope space method does not show an evidence of ecohydrological separation. The lc-excess assessment suggests that the separation seems to occur during the whole study period, which, however, is unlikely in this humid environment. The piecewise isotopes balance method supports the ecohydrological separation with more details in terms of the frequency and duration. The PIB result indicates that the ecohydrological separation does occur in this subtropical humid area. It is more likely to occur in spring and winter. Snowmelt at the study site leads to mixture of the two water pools (mobile and immobile water) and thus the ecohydrological connection. We suggest the ecohydrological separation should be examined with a combination of different methods, aided with high frequency of samplings.

Declaration of Interest Statement:

We declare that we have no conflict of interest, no financial or personal relationships with other people or organizations other than those listed or acknowledged in the manuscript that would influence the publication of our work.

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Figure captions

Fig. 1 Location of study site. The photo in (b) and altitude in (c) were originally extracted from Google Earth, showing some hydrological settings of this site. The red dot symbol in (a), (b) and (c) highlights the study site.

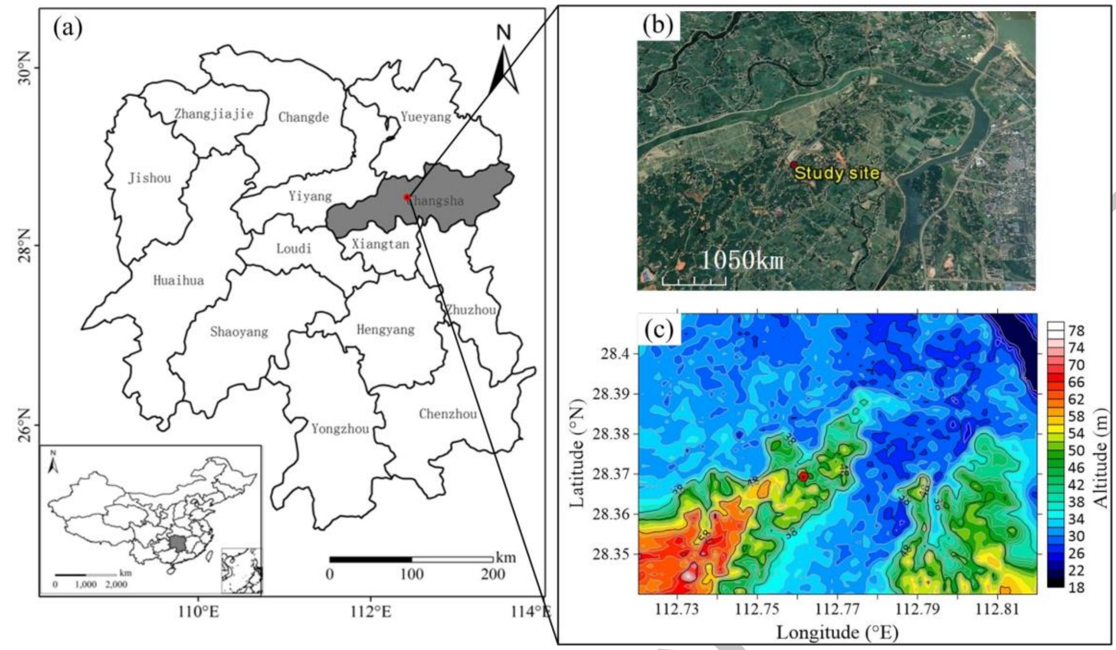
Fig. 2 Water isotopes (δ^2H and $\delta^{18}O$) of precipitation (P), bulk soil water (S), twig xylem water (X) and ground water (G) during the study period. Isotope values are summarized on boxplots for δ^2H (right) and $\delta^{18}O$ (top), respectively. Boxplots show maxima, 75th percentile, average (square), median (solid line), 25th percentile and minima. Different uppercase letters on the top and right of the box indicate mean isotope values are significantly different ($P<0.05$). LMWL represents the local meteoric water line ($\delta^2H= 8.69\times\delta^{18}O+19.99$, $R^2=0.98$).

Fig. 3 Seasonal variations of $\delta^{18}O$ for precipitation, twig xylem water, groundwater (a) and bulk soil water (b), and daily soil water content (c). $\delta^{18}O$ values in (a) and (b) are indicated by colors.

Fig. 4 The lc-excess values of different water pools, showing the ecohydrological separation hypothesis assessment. Boxplots show maxima, 75th percentile, average (square), median (solid line), 25th percentile and minima. Different uppercase letters above the box indicate mean lc-excess values are significantly different ($P<0.05$) while different lowercase letters indicate mean lc-excess values of soil water at different depth are significantly different ($P<0.05$).

Fig. 5 Plant-accessible water replenishment and accumulated precipitation between two consecutive sampling events. Daily average soil water content within the soil profile (θ_a, 0-100 cm) are shown as well. Breakings in red symbol line indicate that no precipitation occurring between the two consecutive samplings. Open circles represent the cases that the difference in twig xylem water $\delta^{18}O$ is very small (<0.2 ‰) between two samplings under the condition of high θ.

Fig. 6 Comparison of three studies on the ecohydrological separation in subtropical humid regions in China, showing the corresponding long-term mean monthly precipitation (1970-2016) and patterns of ecohydrological separation and connection.



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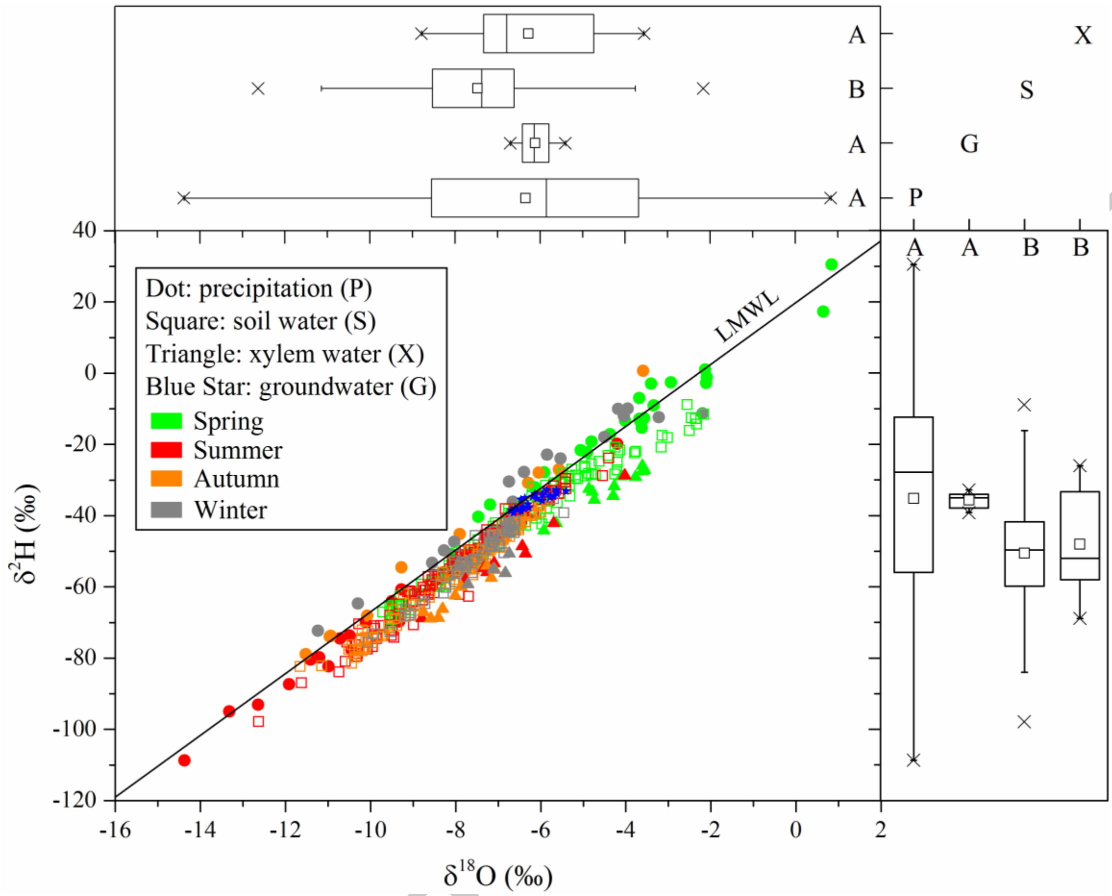

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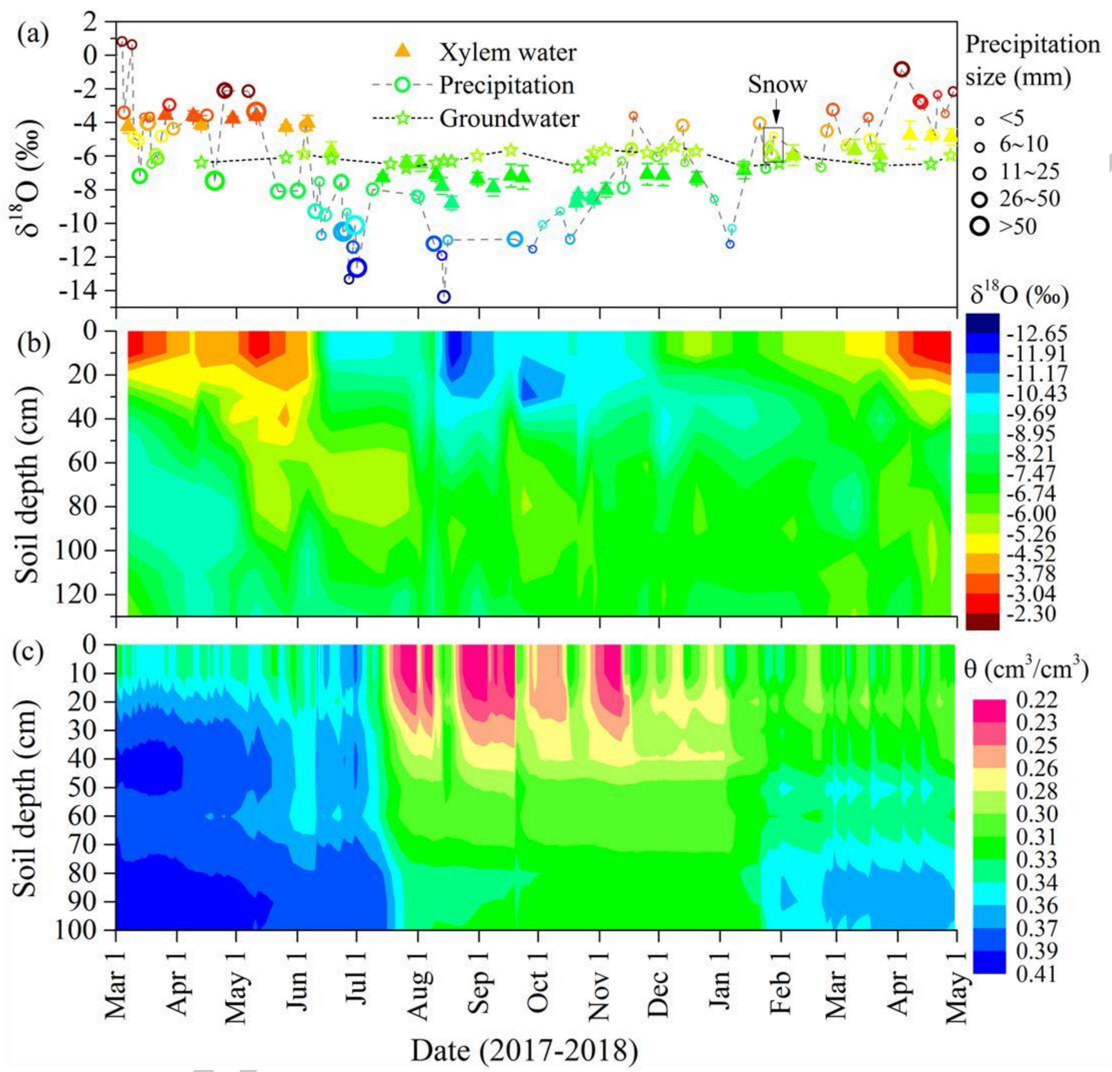

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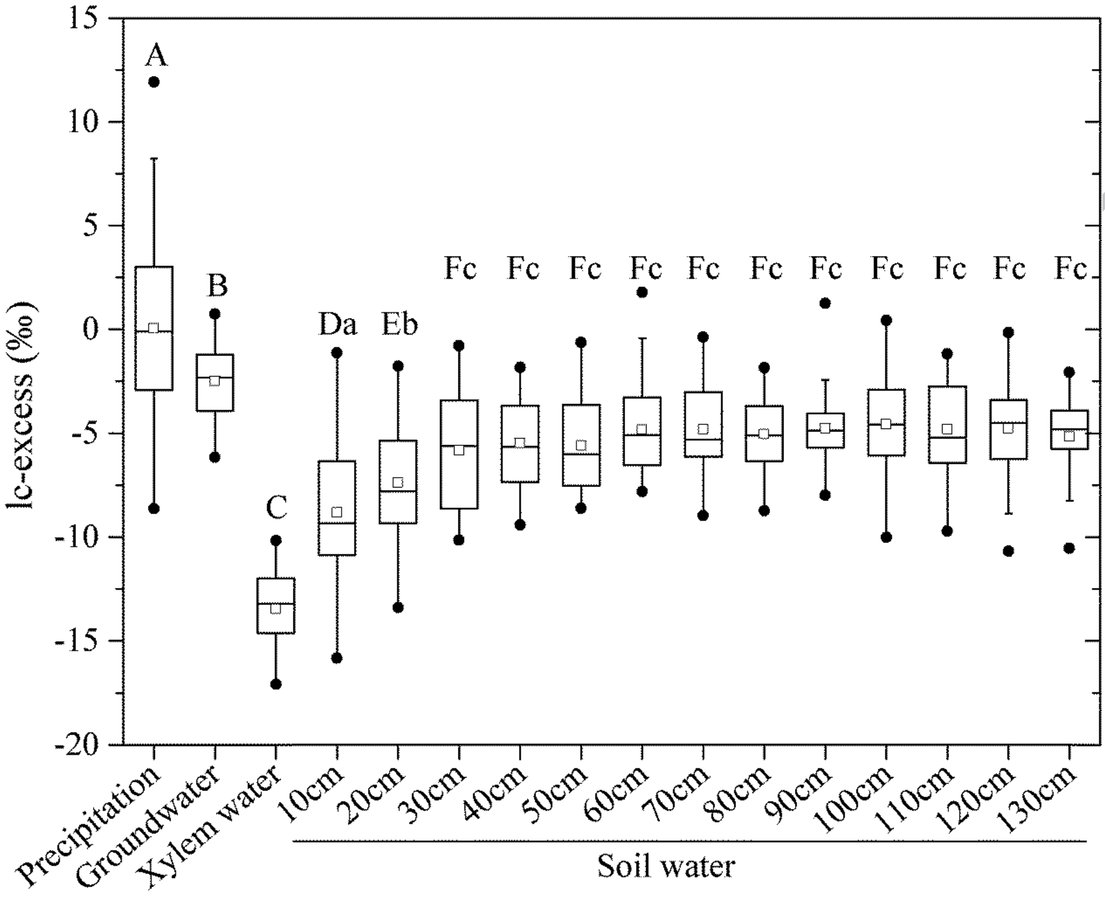

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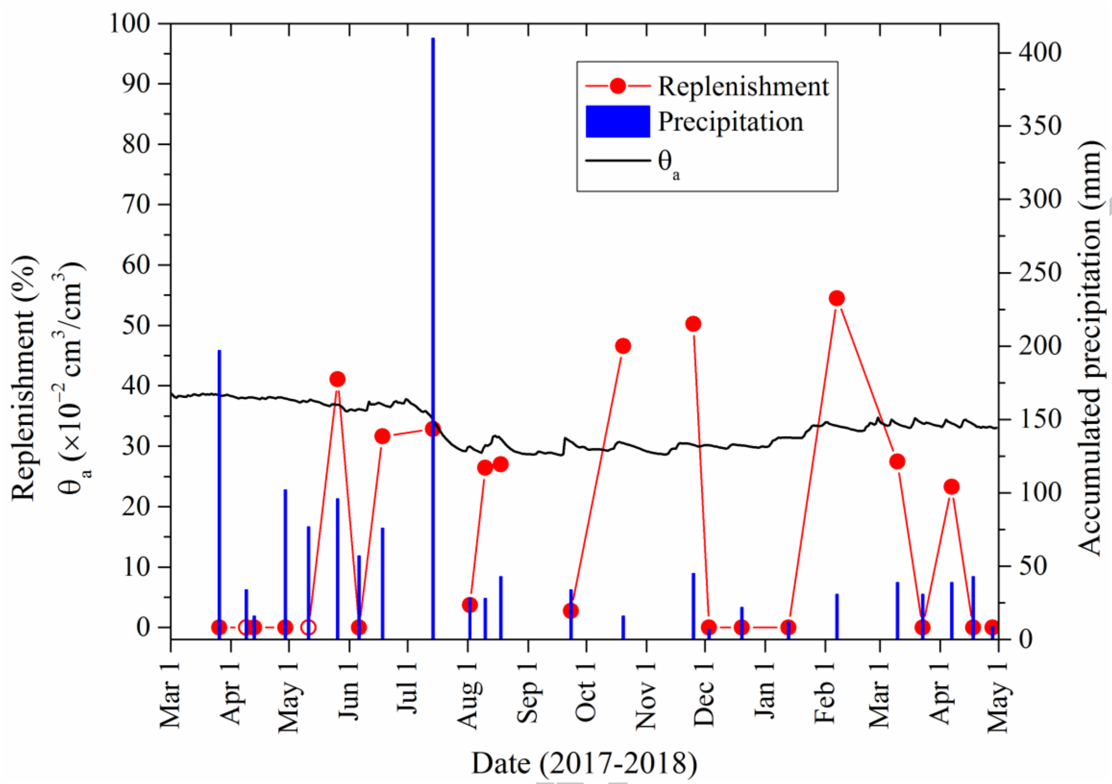

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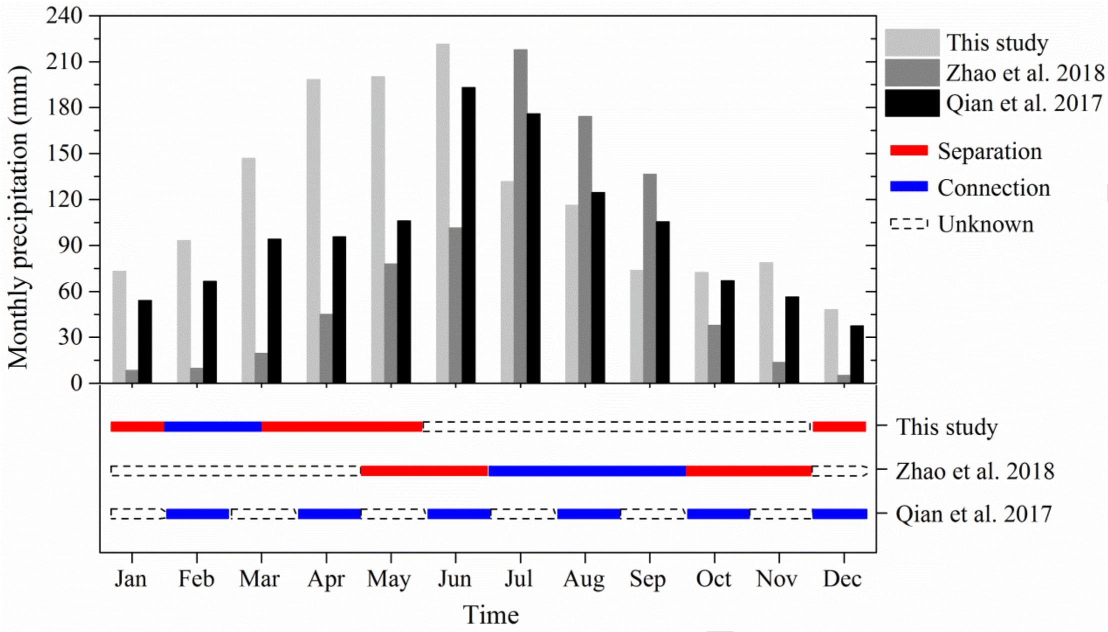

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Highlights

- Two commonly used methods for assessing the ecohydrological separation show inconsistent results.
- A new method based on root zone isotope mass balance supports the ecohydrological separation at the studied humid site.
- The ecohydrological connection occurs during snowmelt at the site.