Progress and challenges in using stable isotopes to trace plant carbon and water relations across scales


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Abstract. Stable isotope analysis is a powerful tool for assessing plant carbon and water relations and their impact on biogeochemical processes at different scales. Our process-based understanding of stable isotope signals, as well as technological developments, has progressed significantly, opening new frontiers in ecological and interdisciplinary research. This has promoted the broad utilisation of carbon, oxygen and hydrogen isotope applications to gain insight into plant carbon and water cycling and their interaction with the atmosphere and pedosphere. Here, we highlight specific areas of recent progress and new research challenges in plant carbon and water relations, using selected examples covering scales from the leaf to the regional scale. Further, we discuss strengths and limitations of recent technological...
developments and approaches and highlight new opportunities arising from unprecedented temporal and spatial resolution of stable isotope measurements.

1 Introduction

Stable isotopes are a powerful tool for tracing biogeochemical processes across spatio-temporal scales (Yakir and Sternberg, 2000). The stable isotope composition of plant material, animal tissues, sediments and trace gases can be used as indicators of ecological change (Dawson and Siegwolf, 2007). The assessment of the circulation of isotopes in the biosphere allows characterisation and quantification of biogeochemical cycles as well as exploration of food webs (Fry, 2006). Stable isotope studies give insights into key reactions of plant metabolism (Schmidt and Gleixner, 1998), can increase our understanding of water movement along the soil-plant-atmosphere continuum (Dawson et al., 1998), and allows palaeoclimatic-physiological reconstructions (Beerling and Woodward, 1998). Moreover, the analysis of the isotopic composition of trace gases exchanged between ecosystems and the atmosphere gives insights in the underlying processes driving the source and sink strength of biomes for CO\textsubscript{2}, CH\textsubscript{4} and/or N\textsubscript{2}O (Flanagan et al., 2005). Stable carbon, oxygen and hydrogen isotope composition of organic matter and inorganic compounds such as CO\textsubscript{2} and H\textsubscript{2}O is altered during vegetation–soil–atmosphere exchange processes, such as evapotranspiration, carbon assimilation and respiration. This leaves an isotopic imprint on soil, plant and atmospheric carbon and water pools and associated fluxes. These isotopic fingerprints can then be used to trace different processes involved in the transfer of carbon and water across the plant–soil–atmosphere continuum. Particularly the multiple-isotope approach, i.e. the simultaneous measurements of stable isotope composition of different elements (\(\delta^2\text{H}, \delta^{18}\text{O} \text{and/or } \delta^{13}\text{C}\), for definition see Tables 1 and 2), provides a unique way to investigate the interrelation between water and carbon fluxes (Ehleringer et al., 1993; Griffiths, 1998; Flanagan et al., 2005; Yakir and Sternberg, 2000). The use of biological archives may enable extrapolation of this information to longer time scales, such as the Anthropocene. Methodological advances allow isotopologue and compound-specific analyses at unprecedented resolution, providing new insight into isotope fractionation processes in metabolic pathways and in biogeochemical processes. Further, a more advanced mechanistic understanding of processes affecting the stable isotope composition in various ecosystem compartments allows modelling and prediction of water and carbon fluxes based on stable isotope information. In turn, new findings open new research frontiers and challenges. Here, we highlight recent progress and developments in carbon and oxygen isotope research and discuss the potential for further extending our knowledge about water and carbon fluxes and cycling. We present advances and challenges on various scales from the leaf (Sect. 2.1), plant (Sect. 2.2), community (Sect. 2.3), ecosystem (Sect. 2.4), and regional scales (Sect. 2.5) as well as of different types of temporal (historical) isotopic archives (Sect. 2.6). At each scale, pertinent reviews are indicated which survey the published literature and pioneering work; thereafter, we focus on selected examples from the last decade. Finally, we highlight strengths and limitations of new technological developments (Sect. 3) and present an outlook (Sect. 4) on what we identify as main goals of the stable isotope research in carbon and water biogeochemistry.

2 Isotope effects across temporal and spatial scales

2.1 Leaf-level processes

2.1.1 CO\textsubscript{2} and H\textsubscript{2}O exchange

Leaf CO\textsubscript{2} and H\textsubscript{2}O fluxes have unique and distinct isotope signals that carry useful physiological and biogeochemical information. For example, environmental stresses, such as drought, cause systematic variation in carbon isotope discrimination during photosynthesis (\(\Delta^{13}\text{C}\), see Table 1), shedding light on different steps of CO\textsubscript{2} transfer from the atmosphere to the chloroplasts (Evans et al., 1986; Farquhar et al., 1989a, b; Brugnoli and Farquhar, 2000). On the other hand, stomatal opening and, hence, transpiration, cause \(^{18}\text{O}\) and deuterium (\(^2\text{H}\)) enrichment of water at the sites of evaporation (\(\Delta_{	ext{ev}}\), see Table 2), which lead to the enrichment of the total leaf water (Dongmann et al., 1974; Farquhar and Lloyd, 1993). The oxygen of leaf-dissolved CO\textsubscript{2} exchanges with the \(^{18}\text{O}\)-enriched leaf water, entraining distinct \(^{18}\text{O}\) discrimination (\(\Delta^{18}\text{O}\)) during photosynthetic CO\textsubscript{2} exchange (Farquhar et al., 1993). The knowledge of \(\Delta^{13}\text{C}\) and \(\Delta^{18}\text{O}\) together can then be used to assess the limitations to CO\textsubscript{2} transfer between the intercellular air space and the chloroplasts (Gillon and Yakir, 2000b).

The theoretical understanding of the individual \(^{13}\text{C}\) and \(^{18}\text{O}\) fractionation phenomena (including transport/diffusion, transformations and exchange processes) in CO\textsubscript{2} and H\textsubscript{2}O is well established for systems in steady-state (Dongmann et al., 1974; Farquhar et al., 1982; Evans et al., 1986; Farquhar et al., 1993). However, we are only at the beginning of gaining theoretical understanding for those in non-steady-state. On-line isotope discrimination studies, i.e. instantaneous measurements of leaf/plant gas exchange and the associated isotopic signals, during transient conditions and short-term dynamics bare the potential to expand our understanding beyond steady-state.

Even though our mechanistic understanding of photosynthetic carbon isotope discrimination and evaporative oxygen isotope enrichment has been increasing within the last decade, there are still open questions and methodological
Table 1. Introduction to terms and equations of carbon isotopes, photosynthetic discrimination and post-carboxylation fractionation.

<table>
<thead>
<tr>
<th>Carbon isotopes, photosynthetic discrimination and post-carboxylation fractionation</th>
<th>Equations</th>
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<tbody>
<tr>
<td>The delta notation for carbon isotopes</td>
<td>$\delta^13X = \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1$ (1)</td>
</tr>
<tr>
<td>Carbon has two stable isotopes, $^{12}C$ and $^{13}C$, with natural abundances of 98.9 and 1.1 %, respectively. The relative abundance of $^{13}C$ in any sample is conventionally expressed in the $\delta$ notation (Eq. 1) which is defined as the relative deviation of the isotope ratio $R$ ($R = ^{13}C / ^{12}C$) of a sample relative to that of an international standard (and is often expressed in ‰). The international standard is the $R$ of CO$_2$ from a fossil belemnite in the Pee Dee formation of South Carolina. Today, $^{13}C$ standards are obtained from the IAEA in Vienna and are referred to as V-PDB (Coplen, 1995, 2011).</td>
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<tr>
<td>Carbon isotope discrimination</td>
<td>$\Delta^{13}C = (\delta^{13}C_a - \delta^{13}C_p)/(1 + \delta^{13}C_p)$ (2)</td>
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<tr>
<td>The change in relative abundance of $^{13}C$ between an educt and product is called discrimination, often denoted with $\Delta$. In the case of CO$_2$ as the source and the plant material as the product of photosynthesis, carbon isotope discrimination is described in Eq. (2).</td>
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<tr>
<td>Photosynthetic carbon isotope discrimination</td>
<td>$\Delta^{13}C = a_0 \frac{p_a - p_s}{p_a} + a \frac{p_e - p_s}{p_a} + (e_0 + a_1) \frac{p_e - p_s}{p_a} + b \frac{p_e}{p_a} + e \frac{R^<em>}{p_a} + f \Gamma^</em>$ (3)</td>
</tr>
<tr>
<td>Isotope discrimination during carbon assimilation has been modelled by Farquhar et al. (1982, 1989a) for C$_3$ plants by Eq. (3). This equation has been developed to describe leaf-level photosynthetic discrimination during the light period, where $e$ denotes the fractionation of mitochondrial respiration in the light, i.e. day respiration (Tcherkez et al., 2010) and $\Gamma^*$, the compensation point in the absence of day respiration. When Eq. (3) is applied to analyse $\Delta^{13}C$ of bulk tissue as an integrative parameter for predicting photosynthetic discrimination during formation of this material, $e$ denotes the integrated respiratory discrimination both during light and dark-respiration. However, additional factors such as discrimination during carbon allocation, tissue turnover or carbon partitioning into different plant organs may affect the observed discrimination. To date, we still lack a quantitative description of these processes (see Sects. 2.1.2 and 2.2).</td>
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<tr>
<td>Simplified model of C$_3$ photosynthetic isotope discrimination</td>
<td>$\Delta^{13}C = a + (b - a) \frac{p_a}{p_a}$ (4)</td>
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<tr>
<td>Few empirical/experimental studies have used Eq. (3), partly due to lack of needed input data. Instead, a simplified version (Farquhar et al., 1982) has been used extensively (Eq. 4). This equation is valid on the condition that effects of boundary layer, internal conductance, photosynthesis, day respiration and allocation are negligible. In strict terms, these conditions are met if boundary layer and internal conductance are infinitely high, photosynthesis and respiration are infinitely low or non-discriminating, and isotope discrimination during allocation and partitioning does not happen. To account for effects of the neglected terms in Eq. (4), the value of $b$ is often slightly reduced ($\approx 28%$) (Brugnoli and Farquhar, 2001).</td>
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<tr>
<td>Intrinsic water use efficiency (WUE$_i$)</td>
<td>$\text{WUE}_i = \frac{A}{g_s} = \frac{p_a (1 - \frac{\delta^{13}C_a}{1.6})}{1.6} = \frac{p_a (1 - \frac{\Delta^{13}C - a}{1.6})}{1.6}$ (5)</td>
</tr>
<tr>
<td>In Eq. (4), $\Delta^{13}C$ is directly proportional to $p_i/p_a$, which is determined by the relationship between photosynthetic assimilation ($A$) and stomatal conductance ($g_s$). Therefore, $\Delta^{13}C$ is a measure of intrinsic water use efficiency WUE$_i$ (or transpiration efficiency), the ratio of assimilation to transpiration, which can be estimated as WUE$_i$ / VPD (Farquhar and Richards, 1984; Farquhar et al., 1989b).</td>
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Table 2. Introduction to terms and equations of oxygen and hydrogen isotopes and evaporative enrichment.

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<td>( \delta X = \frac{R_{\text{sample}} - 1}{R_{\text{standard}} - 1} ) (1)</td>
</tr>
<tr>
<td>Whereas hydrogen has two stable isotopes, (^1\text{H}) and (^2\text{H}) (or (\text{D}:\text{deut}erium), oxygen possesses the isotopes (^{16}\text{O}, {^{17}\text{O}}) and (^{18}\text{O}). Since the natural abundance of (^{17}\text{O}) is very low (approx. 0.038 atom %), mostly the ratio between (^{18}\text{O}) and (^{16}\text{O}) and thus the relative abundance expressed as (\delta^{18}\text{O}) (calculated as shown in Eq. (1) with the standard of Vienna standard mean ocean water (VSMOW) in case of water and VPDB-CO(_2) in case of CO(_2)) is considered.</td>
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Evaporative enrichment

The (evaporative) oxygen (\(\Delta^{18}\text{O}_{\text{ev}}\)) or hydrogen (\(\Delta^{2}\text{H}_{\text{ev}}\)) isotope enrichment of leaf water or plant organic matter is expressed as enrichment above source water (often assumed to be soil or xylem water) by Eq. (6).

Leaf water enrichment

The enrichment of the leaf water has been modelled with approaches of increasing complexity (e.g. Cuntz et al., 2007). Steady-state isotopic enrichment of oxygen or hydrogen over source water at the site of evaporation in the leaf (\(\Delta_s\)) can be calculated by the Craig & Gordon model (Craig and Gordon, 1965; Dongmann et al., 1974) by Eq. (7). In steady state conditions (i.e. source water isotopic composition is equal to the one of transpired water), the isotopic enrichment of water vapour relative to the source water taken up by the plant (\(\Delta_e\)) can be approximated by \(-\varepsilon^+\). This model was developed for open water surfaces and only applies to the water composition at the site of evaporation, and not the whole leaf (mean lamina mesophyll water).

Non-steady-state isotopic enrichment of leaf water

Non-steady-state isotopic enrichment of leaf water (\(\Delta_{\text{LADP}}\)) can be described by correcting Eq. (7) for the so-called Péclet effect (Farquhar and Lloyd, 1993), as shown in Eq. (8). The Péclet effect is the net effect of the convection of unenriched source water to the leaf evaporative sites via the transpiration stream as opposed by the diffusion of evaporatively enriched water away from the sites of evaporation.

Advection-diffusion model of leaf water enrichment

The non-steady-state model of leaf water enrichment as given by Eq. (9) is a simplification of the advection-diffusion description of leaf water enrichment (\(\Delta_{\text{LAD}}\)), as given by Cuntz et al. (2007) and Ogée et al. (2007) in Eq. (10). Steady-state approaches often accurately describe leaf water isotopic enrichment (e.g. Welp et al., 2008), especially for longer times (weeks, months or years) or spatial scales (ecosystem studies). If shorter times and spatial scales are considered (e.g. measurements or gradients across a leaf), non-steady-state approaches are more suitable, especially for modelling leaf water enrichment during the night (Cernusak et al., 2005).

Enrichment of organic matter

Newly produced assimilates are assumed to obtain an imprint of the signature of the average bulk mesophyll leaf water at the time when they were produced. For oxygen, an equilibrium fractionation factor \((\kappa_{\text{eq}})\) results in carboxyl oxygen being ca. 27% more enriched than water (Sternberg and DeNiro, 1983), which has been confirmed for cellulose (e.g. Yakir and DeNiro, 1990), leaf soluble organic matter (e.g. Barnard et al., 2007) and phloem sap sucrose (e.g. Cernusak et al., 2007a, Gessler et al., 2007a).

\(\alpha_{\text{iso}}\) and \(\kappa_{\text{iso}}\) are corresponding to \(\alpha_{\text{H}}\) and \(\kappa_{\text{H}}\) respectively. 

\(\text{Lm}\) is the tortuosity factor of the water path through the mesophyll. The volumetric water content of the mesophyll, and \(\varepsilon\) the ratio of ambient to intercellular vapour pressures.

\(\Delta_{\text{iso}}\) is the isotopic enrichment of water vapour relative to the source water taken up by the plant, and \(\varepsilon_{\text{atm}}/\varepsilon\) is the ratio of ambient to intercellular vapour pressures.

\(\Delta_{\text{LAD}}\) is the isotopic enrichment of leaf water (Craig & Gordon model) (Craig and Gordon, 1965; Dongmann et al., 1974) by Eq. (8). The Péclet effect is the net effect of the convection of unenriched source water to the leaf evaporative sites via the transpiration stream as opposed by the diffusion of evaporatively enriched water away from the sites of evaporation.

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issues which need to be addressed to better understand the physiological information imprinted on plant material.

Progress and challenges

Mesophyll conductance

Mesophyll or leaf internal conductance (often referred to as $g_{m}$ or $g_{i}$) has emerged as a significant (co-)limitation for $CO_{2}$ transport to the chloroplast, with large variation between species and environmental scenarios of light, temperature, drought and salinity (Warren and Adams, 2006; Flexas et al., 2008). On-line measurements of $\Delta^{13}C$ in conjunction with gas exchange have been instrumental in detecting these variations of $g_{i}$. Variation in $g_{i}$ is related to developmental changes and morphological/structural features of leaves, such as cell wall thickness, chloroplast arrangement, and leaf porosity (Flexas et al., 2008; Evans et al., 2009). Moreover, $g_{i}$ may be regulated via the expression of particular aquaporins capable of transporting $CO_{2}$ across plasma membranes (cooporins) (Hanba et al., 2004; Flexas et al., 2007). Strong dynamic responses of $g_{i}$ to various environmental factors at the scale of minutes to days have been reported (Flexas et al., 2008; Bickford et al., 2009), and such variation has also been observed at the canopy-scale (Schäufele et al., 2011). So far, the metabolic basis of these short-term adjustments of $g_{i}$ is unknown.

Contribution of day respiration to $\Delta^{13}C$ dynamics

Recent high-resolution on-line $\Delta^{13}C$ measurements (Bickford et al., 2009) as well as labelling and modelling approaches (Tcherkez et al., 2010) indicate that the isotopic composition of day respiration is not the same as that of concurrently fixed carbon dioxide. In part, the respiratory carbon isotope fractionation during daytime (Tcherkez et al., 2010) is related to fuelling of respiration by old carbon pools (Noguès et al., 2004). This calls for further experimental studies, a more detailed theoretical description of whole-leaf $\Delta^{13}C$ during daytime gas exchange (Wingate et al., 2007; Tcherkez et al., 2004), and consideration of this effect in carbon isotope-based estimations of $g_{i}$.

Water isotope enrichment in leaves

Isotopic enrichment in leaf water is reasonably well understood (Craig and Gordon, 1965; Dongmann et al., 1974; Farquhar and Lloyd, 1993; Cuntz et al., 2007; Ogée et al., 2007), except for the parameter that characterises the effective path length for water movement from the xylem to the site of evaporation (see Pécllet effect, Table 2). This parameter is especially important for modelling leaf water enrichment in non-steady state. Understanding how the effective length is adjusted by environmental conditions requires knowledge of how water transport inside the leaf is changing, for example, with the leaf’s water status (Barbour et al., 2004, 2007; Ferrio et al., 2009; Kahmen et al., 2009; Ferrio et al., 2012). It is likely that in leaves, all water pools are involved during water transport (Yakir, 1998); however, the leaf water pools might not be considered as perfectly mixed (e.g. Helliker and Ehleringer, 2000). Gan et al. (2002) compared different leaf water evaporative enrichment models (i.e. the two-pool model, the Pécllet effect model and the string-of-lakes model), which assume different water isotopic gradients and different mixing of leaf water pools. The different models all described large parts of the observed dynamics of leaf water enrichment but not all facets were captured by a single model. The non-steady state Pécllet model of Farquhar and Cernusak (2005; see Table 2, Eq. 9) is the simplification of the diffusion-advection model of Cuntz et al. (2007; Eq. 10), which assumes that the leaf represents a continuum of unenriched (source) and enriched (evaporative sites) water. The latter, more complex model is less sensitive to noise in the input data and gives smoother results. Cuntz et al. (2007), however, state that comparably good results could also be achieved with different well mixed metabolic pools of water. For epiphytic and non-vascular plants which lack permanent access to soil water, it has been shown that a description of water isotope dynamics requires consideration of distinct water pools as well as water potentials (Helliker and Griffiths, 2007; Hartard et al., 2009; Helliker, 2011).

Exchange of $H_{2}O$ and $CO_{2}$ oxygen isotopes

The enzyme carbonic anhydrase (CA) catalyzes oxygen exchange between water and $CO_{2}$ via (reversible) interconversions of $CO_{2}$ and $H_{2}O$ to bicarbonate ($HCO_{3}^{−}$) and protons (Badger and Price, 1994). This exchange underlies $^{18}O$ discrimination during $CO_{2}$ exchange ($\Delta^{18}O$), and retroflux to the atmosphere of $CO_{2}$ that has previously equilibrated with leaf water, which has a strong effect on the $^{18}O$ content of atmospheric $CO_{2}$ (Farquhar et al., 1993). Also, this signal provides a measure of photosynthetic activity of the terrestrial biosphere (Farquhar et al., 1993). At the leaf level, measurements of gas exchange, CA, $\Delta^{18}O$ and $\Delta^{13}C$ can help to partition mesophyll conductance into a cell wall and a chloroplast component (Gillon and Yakir, 2000a). However, work by Cousins et al. (2008) indicates that CA activity may not be a good predictor for $CO_{2}$-$H_{2}O$ isotopic exchange, endorsing the view that more work is needed to fully understand the control of $^{18}O$ and its physiological implications.

Ternary effects on $CO_{2}$ isotopes during gas exchange

Ternary effects, i.e. effects of concurrent water vapor diffusion on $CO_{2}$ diffusion through stomata, are taken into account for $CO_{2}$ exchange (von Caemmerer and Farquhar, 1981) but not for isotopes. Mesophyll conductance is a parameter greatly influenced by ternary effects. Farquhar and Cernusak (2012) recently showed that by applying the ternary correction, oxygen isotope composition of $CO_{2}$ in the
chloroplast and mitochondria better match the oxygen isotopic composition of water at the sites of evaporation. The ternary effect has been observed to be greatest when the leaf-to-air water vapor pressure deficit is large. Farquhar and Cer- nusak (2012) also observed that a large impact of ternary corrections occurred when the difference in the isotopic composition of CO₂ between the leaf interior and the ambient air was large. The precision of current isotope fractionation models can be improved by applying the ternary correction equations for isotope fractionation and isotope exchange during gas exchange measurements.

2.1.2 Post-carboxylation and respiratory fractionation

The carbon isotope signal, imprinted through photosynthetic ¹³C discrimination (the sum of terms one to four on the right hand side of Eq. 3, Table 1), can be altered by multiple processes in down-stream metabolic pathways (termed post-carboxylation fractionation), which will be reflected in different carbon pools and respired CO₂. Despite early evidence by Park and Epstein (1961), carbon isotope fractionation during dark respiration has long been considered negligible (Lin and Ehleringer, 1997). Systematic studies by Duranceau et al. (1999) and Ghashghaie et al. (2001) with a range of C₃ species again provided clear evidence for substantial and systematic variation in carbon isotope ratios of leaf dark respiration (see review by Ghashghaie et al., 2003). These authors introduced the term “apparent respiratory fractionation” to describe the manifested differences between carbon isotope compositions of leaf dark-respired CO₂ and its putative substrates (mainly carbohydrates), caused by multiple processes in the respiratory pathways (see below). The work of Ghashghaie and coworkers promoted a significant number of studies on post-carboxylation fractionations in down-stream metabolic processes (Klumpp et al., 2005; Badeck et al., 2005; Cernusak et al., 2009; Tcherkez et al., 2011; Werner and Gessler, 2011).

Progress and challenges

Post-carboxylation fractionation

Already within the Calvin cycle, isotopic fractionation occurs mainly due to metabolic branching points and the use of triose phosphates that can either be exported to the cytosol or continue to be used within the Calvin cycle. The triose phosphates that are not exported are subject to certain enzyme catalyzed reactions (aldolisation and transketolisation) which involve position-specific discrimination during C–C bond making. As a result, the C-3 and C-4 positions within glucose are enriched in ¹³C and thus a non-homogeneous intra-molecular distribution of ¹³C within carbohydrates is established (Rossmann et al., 1991; Tcherkez et al., 2004; Gilbert et al., 2009). Subsequently, photorespiration and starch–sucrose partitioning result in diel changes in isotopic signatures of phloem sugars, with day sucrose being ¹³C-depleted, while night exported sucrose is ¹³C-enriched (Tcherkez et al., 2004; Gessler et al., 2008). Analyses of sugar δ¹³C and its diurnal variations offer potential for improved tracing of these changes in these metabolic activities.

Apparent respiratory fractionation

During the last decade the knowledge of relevant apparent fractionation in the respiratory pathways has significantly advanced, demonstrating substantial variability in respiratory fractionation among species, organs and functional groups, as well as with environmental conditions (see reviews by Ghashghaie et al., 2003; Werner and Gessler, 2011). The observed apparent respiratory fractionation and its variability are mainly attributed to (i) non-homogeneous ¹³C-distribution within hexose molecules reported by Rossmann et al. (1991) and modelled by Tcherkez et al. (2004), (ii) relative contributions of different pathways to respiration (reviewed by Ghashghaie et al., 2003), as well as (iii) enzymatic isotope effects during decarboxylation reactions (recently reviewed by Tcherkez et al., 2011).

Fragmentation fractionation

The non-homogeneous intra-molecular distribution of ¹³C in carbohydrates, results in so-called “fragmentation fractionation” (Tcherkez et al., 2004), leaving its imprint on synthesized metabolites. If one of these products is decarboxylated, then respired CO₂ will carry an isotopic signature different from the average sugar signature. New data indicate that the heterogeneous ¹³C distribution in carbohydrates may vary among species and with environmental conditions (Gilbert et al., 2011). Moreover, switches between substrates (Tcherkez et al., 2003) during light–dark transition of leaves (i.e. light enhanced dark respiration due to decarboxylation of light-accumulated malate, Barbour et al., 2007) and the oxidative pentosephosphate pathway (PPP) were shown to markedly change δ¹³C, of respired CO₂ (Bathellier et al., 2008, 2009). The implication of these processes still needs to be explored.

Temporal dynamics and apparent respiratory fractionation

So far, a full quantitative understanding of apparent respiratory fractionation has not yet been achieved. However, measurements with a high temporal resolution indicated remarkable diel dynamics in leaf respiratory δ¹³CO₂, which differed between functional plant groups (Priault et al., 2009; Werner et al., 2009; Werner and Gessler, 2011). Feeding experiments with positionally labelled glucose or pyruvate can trace changes in carbon partitioning in the metabolic branching points of the respiratory pathways (Tcherkez et al., 2004), which has been used at the organ level as evidence for an important contribution of PPP to root respiration (Bathellier et al., 2009) as well as to identify differences between
Functional groups (Priault et al., 2009; Wegener et al., 2010). One challenge of labelling experiments is to find methods for channelling additional labelled metabolites into plant organs in vivo to shed further light on potential involvement of these metabolites and their metabolic pathways. Furthermore, the commitment of metabolites to alternative pathways at metabolic branching points needs to be quantified. This is particularly relevant where metabolic channelling evokes compartmentation in organelles with membranes, which are impermeable for intermediate products, as shown recently for the Krebs cycle (Werner et al., 2011).

Intra-molecular site-specific isotope fractionation

Recent results of Gilbert et al. (2011) demonstrated that access to site-specific isotope fractionation is now possible using $^{13}$C NMR (see Sect. 3) to directly determine intramolecular $^{13}$C distributions at natural abundance. The challenge in the application of this new technology is to ensure sufficient sample quantities from metabolic pools of interest. Of special interest is that obtained data can be interpreted directly in terms of isotope effects associated with specific enzymes.

Environmental effects

More studies of the sensitivity of respiratory $\delta^{13}$C to changes in environmental conditions and between organs are needed, which will allow for a better understanding of temporal variability in post-photosynthetic fractionation (Dubbert et al., 2012) and could provide a basis for the use of respiratory $\delta^{13}$CO$_2$ as an indicator of physiological activity (e.g. Barbour et al., 2011a, b).

2.1.3 Bulk leaf tissue $\delta^{13}$C and $\delta^{18}$O and water use efficiency

The $\Delta^{13}$C model by Farquhar et al. (1982; Eq. 3 in Table 1) predicts a linear relationship between $\Delta^{13}$C and intrinsic water use efficiency (WUE$_i$, the ratio of net assimilation, $A$, to stomatal conductance, $g_s$), for conditions where mesophyll conductance is very high and (photo)respiratory $^{13}$C discrimination is negligible (Eq. 4). Empirical studies in controlled conditions confirmed this linear relationship between $\Delta^{13}$C, estimated from bulk biomass carbon isotope composition ($\Delta^{13}$C$_b$), and WUE$_i$ (Farquhar et al., 1982, 1989b; Ehleringer et al., 1993; Griffiths, 1998; Brugnoli and Farquhar, 2000). In the following three decades, this linear (simplified) model of $\Delta^{13}$C (Eq. 4) was used widely as an indicator of water use efficiency at the leaf, plant and ecosystem scale (e.g. Bonal et al., 2000; Lauteri et al., 2004; Saurer et al., 2004; Ponton et al., 2006) in retrospective studies of carbon–water relations based on biological archives (see Sect. 2.6), and in breeding crop varieties for improved yield under water-limited conditions (Condon et al., 2002).

Progress and challenges

The dual-isotope approach

Combined analyses of the carbon and oxygen isotopic composition of bulk leaf biomass provide a means to distinguish the separate effects of stomatal conductance and net photosynthesis on WUE$_i$ (Scheidegger et al., 2000). Preferably, however, carbon isotope discrimination $\Delta^{13}$C$_b$, and bulk biomass oxygen isotope discrimination, $\Delta^{18}$O$_b$, should be used in such an approach to account for effects of differences in $\delta^{13}$C of assimilated CO$_2$ and variations of $\delta^{18}$O of source water. A distinction between stomatal and photosynthetic influences cannot be made from analysis of $\Delta^{13}$C$_b$ alone. The conceptual model of Scheidegger et al. (2000) was successfully applied in the field (Keitel et al., 2003, Sullivan and Welker, 2007) and further adapted for air pollution studies evaluating the effect of NO$_x$ on plant metabolism (Siegwolf et al., 2001; Guerrieri et al., 2009, 2010; Savard, 2010). Grams et al. (2007) extended the model to estimate stomatal aperture directly for interpreting physiological changes. $\delta^{18}$O of bulk organic matter has also been used to determine whether a change in WUE$_i$ results from the increase in atmospheric CO$_2$ (Saurer and Siegwolf, 2007). Also, the effects of changes in vapor pressure deficit (VPD) resulting from increasing temperature or decreasing precipitation have been assessed along a Siberian transect (Sidorova et al., 2009; Knorre et al., 2010). The dual-isotope approach has proven a valuable concept for ecological applications. However, the interpretation of $\Delta^{13}$C$_b$ in terms of WUE$_i$ under natural changing environments is complex (e.g. Seibt et al., 2008), as it provides a time-integrated record of photosynthetic discrimination over the period that the carbon forming the leaf was fixed, which can be derived from multiple sources, e.g. fresh assimilates, carbon exported from mature leaves or even older storage pools. Moreover, different leaf carbon pools have different residence and turnover times (Nogués et al., 2004; Lehmeier et al., 2008, 2010b). Thus, during leaf formation, growth and maintenance leaf bulk material integrates isotopic information from different time periods and sources, which is weighted by the amount of carbon incorporated from each source/period. Therefore, interpretation of $\Delta^{13}$C$_b$ in terms of WUE$_i$ under natural changing environments requires several precautions, as described below.

Interpretation of $\Delta^{13}$C$_b$ and $\Delta^{18}$O$_b$ in relation to leaf traits

A comparison of WUE$_i$ between different species based on $\Delta^{13}$C in bulk leaf material is nontrivial. Differences in not only leaf structural, anatomical, but also physiological traits can modulate $\Delta^{13}$C$_b$ (Ehleringer, 1993; Werner and Mágua, 2010), as well as $\Delta^{18}$O in lamina leaf water (Barbour and Farquhar, 2003; Kahmen et al., 2008) and $\Delta^{18}$O$_b$. Different leaf structures may affect mesophyll conductance (e.g.
Kogami et al., 2001; Hanba et al., 2003) and thus $\Delta^{13}C_b$ (see Sect. 2.1.1). Mesophyll conductance is generally neglected when calculating WUE$_l$ from stable isotope discrimination (see Eq. 5). If there are varying influences of mesophyll conductance on $\Delta^{13}C$ among species, WUE$_l$ calculated from Eq. (5) will be not directly comparable. Leaf traits may also affect the scaled effective path length for water movement from the xylem to the site of evaporation (Wang and Yakir, 1995) and thus influence $\Delta^{18}O_b$ (cf. Eq. 8, Table 2). The conceptual model of Scheidegger (2000) does not account for such effects but strictly assumes oxygen isotope enrichment to be only affected by the ratio of ambient to intracellular water pressure ($e_a/e_i$; cf. Eq. 7). Any other factor varying leaf water evaporative enrichment and thus $\Delta^{18}O_b$ will thus constrain the interpretation of the impact of stomatal conductance versus net photosynthesis on WUE$_l$. Moreover, due to differences in phenological phases and length of growing period leaf $\Delta^{13}C_b$ and $\Delta^{18}O_b$ of co-occurring species might provide an integrated signal over diverging environmental conditions (e.g. Werner and Mágas, 2010). Thus, species-specific differences in phenology, growth pattern and leaf structures might constrain a direct comparison of bulk leaf $\Delta^{13}C$ and $\Delta^{18}O$ between different species (Hanba et al., 2003; Warren and Adams, 2006). Moreover, ontogeny can markedly alter the isotopic signature (Terwilliger et al., 2001; Bathellier et al., 2008; Salmon et al., 2011). Repetitive sampling and isotope analysis of tissues and compounds which are known to integrate shorter and more defined time periods such as phloem sugars (e.g. Keitel et al., 2003, Dubbert et al., 2012) during the growing season, together with bulk leaf assessments, might help to better constrain the physiological meaning of $^{13}C_b$ and $^{18}O_b$. For community scale stable isotope approaches (see Sect. 2.3), the potential limitations of using the bulk isotope signature need to be kept in mind and sampling strategies need to be adapted. For trees, the intra-annual analysis of tree ring, whole wood or cellulose can provide a tool to study periods during the growing season when the isotopic signature in this archive is directly coupled to leaf physiology (Helle and Schleser, 2004; Offermann et al., 2011).

Interpretation of leaf $\Delta^{13}C_b$ and $\Delta^{18}O_b$ in relation to storage and remobilization

A part of the leaf structural organic matter of deciduous trees is made from remobilized starch (or other non-structural compounds) from overwinter storage pools in stems and roots (Kozlowski and Pallardy, 1997). That material was derived from the photosynthetic activity of previous year leaves, with different morpho-physiological characteristics in other environmental conditions, producing a previous year isotopic signal. Since starch can be $^{13}C$-enriched by up to 4% as compared to newly assimilated sugars (Gleixner et al., 1998), growing leaves supplied from storage pools are often strongly $^{13}C$ enriched in spring (e.g. Terwilliger, 1997; Helle and Schleser, 2004). Moreover, during starch breakdown, carbonyl oxygen atoms are exchanged with unenriched water in stems, causing these incorporated starch-derived sugars to be $^{18}O$ depleted as compared to sugars formed in transpiring leaves (Gessler et al., 2007b). This “isotopic starch imprint” in the newly developed leaves is thought to be diluted during the growing season by carbon turnover and the incorporation of new assimilates into bulk leaf organic matter (see e.g. the seasonal course of bulk leaf $\delta^{13}C$ of beech shown by Helle and Schleser, 2004). For interpreting the isotopic composition of a deciduous leaf, it is thus important to consider when the leaf was harvested during the season and that there might be species-specific differences in the extent to which the starch imprint or the influence of the assimilates incorporated during the current growing season dominate the bulk isotope signal. In turn, coniferous needles can accumulate large amounts of starch in spring, followed by mobilization towards the summer, and starch contents are generally low during the winter (e.g. Ericsson, 1979). As a consequence, at least part of the $\Delta^{13}C_b$ during the growing season is related to the variation of starch content and isotopic composition (Jäggi et al., 2002), a fact that also needs to be taken into account when calculating WUE$_l$ from $\Delta^{13}C_b$ and comparing it among species.

Thus, the complexity of processes influencing $\Delta^{13}C_b$ may constrain its use in ecological field studies. Carbon pools with shorter turnover times and thus a better-defined origin such as leaf soluble sugars (Brugnoli and Farquhar, 1988), phloem allocated carbon (e.g. Gessler et al., 2004; Scartazza et al., 2004) or even dark-respired $\delta^{13}CO_2$ of recent photosynthates (Barbour et al., 2011b) are therefore better indicators for recent changes in WUE$_{ia}$, as outlined below (Sect. 2.2).

2.2 $^{13}C$ and $^{18}O$ isotopes to trace plant integrated processes and plant-soil coupling

With their pioneering work on the phloem carbon isotopic composition of grasses (Yoneyama et al., 1997) and trees (Pate and Arthur, 1998), two groups paved the way to getting temporally and spatially (whole canopy) integrated information on leaf physiology. Pate and Arthur (1998) applied natural abundance stable isotope approaches to investigate transport and allocation of assimilates by combining sampling of phloem sap organic matter and source and sink organs. This work focused on transport directions and patterns of source-to-sink transport, while tracing of carbon allocation with high temporal resolution in plants required the use of labelling experiments (e.g. Hansen and Beck, 1990).

Within the last ten years it was, however, shown that the transport of newly assimilated carbon within the plant and from the plant to the rhizosphere can also be followed by natural abundance stable isotope techniques (e.g. Scartazza et al., 2004; Brandes et al., 2006; Wingate et al., 2010b).
Progress and challenges

Plant integrating information and phloem transport

The δ^{13}C of phloem organic matter is increasingly being used to derive information on carbon allocation, canopy integrated WUE and canopy integrated mesophyll conductance in plants affected by environmental conditions (e.g. Keitel et al., 2003; Gessler et al., 2004; Scartazza et al., 2004; Barbour et al., 2005; Rascher et al., 2010; Ubierna and Marshall, 2011; Dubbert et al., 2012). A dual-isotope approach (δ^{13}C and δ^{18}O see Sect. 2.1.3) can also be successfully applied to phloem sugars to distinguish whether net assimilation and/or stomatal conductance is changing as a result of environmental conditions (Keitel et al., 2003, Cernusak et al., 2003, 2005; Brandes et al., 2006; Keitel et al., 2006). Even though the carbon and oxygen isotope composition of phloem organic matter can, in principle, integrate leaf physiology over the whole canopy and track transport of assimilates within the plant, it is now clear that several uncertainties constrain the interpretation of phloem isotopic information. These are related to (i) the temporal integration of the isotope signal in the phloem, (ii) potential changes of the isotope composition of phloem sugars in basipetal direction, and (iii) the chemical composition of phloem transported organic matter.

Phloem sugars and temporal integration

Short-term variations in the isotopic composition of leaf sugars – induced by either an environmental signal or plant internal processes – might or might not be reflected in the isotopic composition of phloem organic matter. Twig phloem organic matter of trees (e.g. Gessler et al., 2007a) and the stem phloem of herbaceous species (e.g. Gessler et al., 2008) can be applied to monitor diel variation of evaporative δ^{18}O and δ^{2}H enrichment or carbon isotope fractionation. In the trunks of adult trees, however, the mixing of sugars of different metabolic origins can dampen the short-term variations and the isotope signatures provide time-integrated information on canopy processes instead (Keitel et al., 2006; Rascher et al., 2010).

2.2.1 Change of the original isotope signal in phloem sugars

The original isotope signal imprinted on sugars in the leaf may be altered during basipetal transport in the phloem of trees (e.g. Rascher et al., 2010). The transport of sugar molecules itself does not fractionate to a measurable extent. However, carbon fixation by PEPc in the bark and oxygen atom exchange with stem water during metabolic processes in the stem tissue together with the continuous unloading and loading of sugars from and to the phloem might contribute to the observed isotope patterns (Barnard et al., 2007; Gessler et al., 2009). The change in δ^{13}C along the transport path, however, varies strongly among species ranging from δ^{13}C enrichment (Brandes et al., 2006; Wingate et al., 2010a) and no change in δ^{13}C (Pate and Arthur, 1998; Gessler et al., 2007a) to δ^{13}C depletion (Rascher et al., 2010). The nature of these species-specific differences remains to be clarified and might shed new light on mechanisms controlling assimilate partitioning in trees.

Chemical composition of phloem sugars

It is often assumed that only one major sugar, namely sucrose, is present in the phloem. However, besides sucrose, there are other transport carbohydrates – depending on species and phloem loading mechanisms – such as myo-inositol and raffinose family sugars (Karner et al., 2004). In addition, it is still a matter of debate if hexoses are transported in the phloem or not (van Bel and Hess, 2008; Liu et al., 2012). Phloem sugar composition can vary with environmental conditions, which could be one factor for changes in phloem δ^{13}C (Merchant et al., 2010), independent of the original leaf-borne isotope signal, since δ^{13}C differs between different carbohydrate molecules (Schmidt, 2003; Devaux et al., 2009). Compound-specific analysis, provided by modern LC- and GC-IRMS techniques (Sect. 3), will help to differentiate between changes in phloem δ^{13}C that result from either changes in the chemical composition or changes in leaf level fractionation. In addition, comparable methods should be used to characterise the compound-specific oxygen isotope composition of phloem organic matter.

Only recently, the natural abundance stable isotope information in soil and ecosystem respired CO_2 cross-correlated to photosynthesis (or its proxies) has been used systematically to characterise the speed of link between canopy and soil processes (see review by Kuzyakov and Gavrichkova, 2010; Wingate et al., 2010b). Even though such approaches have significant potential, there is still debate about the physiological information conveyed by the isotope signal and of the processes involved (see review by Brüggemann et al., 2011).

The link between above- and belowground processes

In their review, Kuzyakov and Gavrichkova (2010) postulated that approaches which quantify time lags between proxies of photosynthetic activity and natural abundance δ^{13}C in soil respiration are (besides other techniques) appropriate to study the link between above- and belowground processes. Mencuccini and Hølttø (2010) reviewed different approaches to assess the speed of link between assimilation and soil respiration and concluded, in contrast, “that isotopic approaches are not well suited to document whether changes in photosynthesis of tall trees can rapidly affect soil respiration”. These different opinions may be related to uncertainties on the mechanisms involved, as described by Kayler et al. (2010a, b): on the one hand, there is evidence that pressure–concentration waves (Thompson and
Holbrook, 2004), which travel rapidly through the phloem of plants (and not the supply of new assimilates transported via the phloem to the roots and the rhizosphere), are responsible for the fast response of soil respiration to changes in photosynthesis. On the other hand, the time-lag between the fixation of a carbon molecule during photosynthesis and its respiration belowground may contain real and important information about plant physiology and carbon use as well as the degree to which plant and soil are coupled (Kayler et al., 2010a). This information may be obtained by the assessment of δ13C in respired CO2 but also in respiratory substrates when points listed above are taken into account. To unravel the importance of the different relevant processes, we need novel, pertinent experiments which combine (a) continuous measurements of the natural abundance stable isotope composition of soil respired CO2, as done by Wingate et al. (2010b), with (b) appropriate statistical approaches that are able to track time lags between photosynthesis and soil CO2 efflux, as applied by Vargas et al. (2011). Whereas (a) indicates how long it takes until a molecule with a given isotope composition imprinted during photosynthesis is transported from the leaves via the phloem to the roots where it is respired, (b) allows to detect the response time(s) of soil respiration towards changes in carbon assimilation, which might or might not be faster than the transport of a given molecule from the canopy to belowground.

2.3 Community-scale processes

Because different species living within the same habitat show marked differences in the isotopic composition of their leaf tissues, characterising community-wide variation in δ13C and/or δ18O, can provide potentially powerful tools for investigating the physiological basis for niche partitioning among community members (Dawson et al., 2002). Good examples are utilization of different water sources and redistribution (Caldwell et al., 1998; Ryel et al., 2003), which can in turn be linked to community composition (Ehleringer et al., 1991), niche partitioning and spatial and temporal variations in plant distributions (e.g. Dawson et al., 2002; Snyder and Williams, 2000; Stratton et al., 2000; Drake and Franks, 2003; Rose et al., 2003; Grams and Matyssek, 2010). As stated above (Sect. 2.1.3), it must be kept in mind when comparing different species that the isotopic composition of bulk leaf material might be influenced by multiple factors such as structural, anatomical and physiological traits but also phenology. There are only very few community-wide investigations on this topic (see Smedley et al., 1991; Guehl et al., 2004; Kahmen and Buchmann, 2007). This scarcity may be in part related to difficulties in assigning cause and effect to observed variation from either physical (e.g. water availability, light) or biological (e.g. resource competition) factors.

Progress and challenges

Adding dual-isotope approaches to community ecology

13C and 18O signals can trace biotic and abiotic interactions within the plant community and may contribute to identifying what shapes community-scale processes. However, individual plants will not necessarily respond to environmental perturbations as “a community”, but may respond according to species-specific traits and requirements and additionally depend on the interactions with the surrounding environment and other present species (e.g. Roscher et al., 2004; Gubsch et al., 2011). Competition and/or facilitation interactions between species, e.g. through depletion of a particular resource, may also be a source of isotopic variation, as shown for plant–plant competition for above- and belowground resources by combining δ13CO2 and δ18H2O analyses (Ramírez et al., 2009; Grams and Matyssek, 2010). Moreover, changes in community functioning, for example by alterations in nutrient, carbon and hydrological cycles after exotic plant invasion, can also be traced through stable isotopes (e.g. Rascher et al., 2011).

Tracing spatial interaction between species within plant communities

Spatio-temporal variations in isotope ratios (i.e. isoscapes) contain a potential wealth of information regarding ecological processes (West et al., 2008; Bowen et al., 2009), which have, so far been applied at larger spatial scales (see Sect. 2.5). At the community scale, spatial heterogeneity in resource availability, differential resource utilisation by neighbouring species and their interactions (competition and facilitation) occur in a spatially explicit dimension, which may contain crucial information regarding community functioning. For example, hydraulic redistribution of water sources is a key process which can shape plant communities (see review by Prieto et al., 2012). Recently it has been shown that downscaling isoscapes to the community level allowed tracing the spatial impact of an invasive species on community functioning (Rascher et al., 2012), and may therefore open new possibilities in resolving the spatial component of within-community interactions.

Tracing functional groups/community composition

During the last decade, the functional group approach has proved to be an efficient way to analyse plant functioning at the community scale. Leaf bulk Δ13C allows the distinction of broad plant functional types, differing in structural, phenological and physiological leaf traits (Brooks et al., 1997; Bonal et al., 2000; Werner and Mágua, 2010). Functional traits such as water or nutrient use strategies, carbon acquisition, growth behaviours, and phenological cycles contribute significantly to the observed variation in isotope composition (e.g. Warren and Adams, 2006; Gubsch et al., 2011; Salmon
et al., 2011; Ramírez et al., 2012). However, the responsiveness of leaf $\Delta^{13}$C as a functional tracer has to be verified for different communities and may differ with the predominant environmental constraints for plant growth and survival (e.g. Caldeira et al., 2001). For example, in a tropical rainforest, $\Delta^{13}$C was associated with differences in shade tolerance (Bonal et al., 2000; Guehl et al., 2004), whereas in an upland water-limited grassland of Greece, a semi-arid Inner Mongolian steppe, and a Portuguese Mediterranean macchia grouping according to $\Delta^{13}$C was associated with species’ competitive ability related to WUE, nitrogen use efficiency, and structural adaptations to drought (Tsialtas et al., 2001; Gong et al., 2010; Werner and Máguas, 2010).

The role of water source partitioning on community functioning

Several mixing models have been used to determine the contribution of different water sources to plant and ecosystem evapotranspiration: Linear mixing models can be applied if the differences of $\delta^{18}$O among the water sources and xylem plant water are large enough; $\delta$D-$\delta^{18}$O plots can be used if the difference between water sources and xylem water is small (Ogle and Reynolds, 2004; Dawson and Simonin, 2011). The use of multiple source mass balance analyses can improve the capacity to quantitatively and objectively evaluate complex patterns in stable isotope data for determining possible contributions of different sources to total plant water uptake (see review by Hu et al., 2009). Furthermore, combining water source partitioning with indicators of species functional responses (e.g. changes in leaf water potential and carbon isotope discrimination) lent insight regarding the degree of plasticity among individual members of a given plant community (Máguas et al., 2011). However, there is increasing awareness that the utilisation of simple linear mixing models to infer plant water uptake by comparing $\delta$D and $\delta^{18}$O of xylem or root crown, on the one hand, and soil water, on the other hand, does not adequately reflect the high heterogeneity of water sources that may be available for a plant. Given the importance of resource variability at the community level, the utilisation of more complex mixing models (for example, by Phillips, 2001; Phillips and Gregg, 2001, 2003; Parnell et al., 2010) as well as Bayesian models (Ogle et al., 2004) may be fruitful.

2.4 Use of stable isotopes to disentangle ecosystem exchange processes

At the ecosystem scale, stable isotopes can provide insight into the complex interaction between vegetation, soil and atmosphere exchange of carbon and water fluxes, including their responses and feedbacks to environmental drivers (e.g. Flanagan and Ehleringer, 1998; Dawson et al., 2002; Yakir and Sternberg, 2000; Yakir, 2003, Hemming et al., 2005; please see Bowling et al., 2008 for review of pioneer and recent literature). The core of the variation behind patterns in $\delta^{13}$C of ecosystem respiration ($\delta^{13}$C_R) lies in photosynthetic discrimination, the magnitude of metabolic fluxes and several post-carboxylation fractionation processes that differ between autotrophic and heterotrophic organs (see Sect. 2.1.2 and references therein). How these components manifest into integrative measures such as ecosystem respiration is fundamental to understanding ecosystem physiology and biogeochemistry. It is clear that ecosystem responses to climate and land use change, or perturbations, such as drought or fire, are an integrative signal from a network of carbon pools and organisms linking legacy conditions to current observations (e.g. Buchmann et al., 1997a, b, 1998; Ehleringer et al., 2000). Thus, to properly account for ecosystem trace gas exchange and partitioning by stable isotopes, a detailed knowledge of the physical and biological basis of the isotopic signals for each of the fluxes and their dynamics across spatial and temporal scales in soil–biosphere–atmosphere interactions is required.

Progress and challenges

Recent findings on component sources and fluxes

Previous ecosystem $^{13}$C and $^{18}$O isotope research primarily focused on partitioning of soil and canopy sources, which are now a mainstay of ecosystem isotopic investigations (e.g. Buchmann et al., 1998; Kaplan et al., 2002; Yakir and Sternberg, 2000 and literature therein). The inherent complexity behind ecosystem respiration lies behind the many contributing sources. Nowadays, studies of these components have expanded to include stem CO$_2$ flux, mycorrhizal and microbial contributions (Esperflichshütz et al., 2009), litter decomposition (Bird et al., 2008; Rubino et al., 2010), dissolved organic carbon (Sanderman and Amundson, 2008; Müller et al., 2009), erosion (Schaub and Alewell, 2009), soil organic matter dynamics (Klump et al., 2007; Kayler et al., 2011) and CO$_2$ storage in soil air and solution (Gamnitzer et al., 2011). Labelling has also played a central role in achieving a higher level of certainty in observing single source temporal patterns (Ubierna et al., 2009; Powers and Marshall, 2011). Similarly, the water oxygen and hydrogen isotope composition has been used as natural or artificial tracer of the ecosystem and component water fluxes and to partition evaporation and transpiration (e.g. Yakir and Wang, 1996; Yepez et al., 2005, 2007; Williams et al., 2004; Lai et al., 2006; Rothfuss et al., 2010; Wang et al., 2010; Kim and Lee, 2011) to assess ecosystem water use efficiency (WUE) (Ponton et al., 2006) and, e.g. the effects of hydraulic redistribution by roots and mycorrhiza (e.g. Ludwig et al., 2004; Kurz-Besson et al., 2006; see Sect. 2.3.2). These detailed studies are important because inferences can be drawn concerning carbon and water dynamics at larger time scales (e.g. erosion, soil organic matter transformations), and spatial variability across the ecosystem can be better described. The
advantage of these studies is two-fold: (1) underlying connections between ecosystem carbon pools and fluxes and the influence of changes in environmental drivers can be characterised, and (2) results can be used in models designed to partition ecosystem respiration.

Canopy labelling

Whole ecosystem dynamics studied in situ using isotopes, at first pioneered through girdling (Högberg et al., 2001), have increased in number through whole canopy tracer application. Advances in our understanding of ecosystem processes through canopy labelling include assessing photosynthetic-soil-respiration coupling strength (Steinmann et al., 2004; Högberg et al., 2008; Bahn et al., 2009; Gannitzer et al., 2009, 2011), carbon allocation patterns (Kuptz et al., 2011; Epron et al., 2011), and shading impacts (Warren et al., 2012), to name a few. Quantitative methods of canopy labelling in connection with on-line tracer measurement techniques (Sect. 3) and modelling of the tracer distribution data (e.g. by compartmental analysis), holds the promise of testing hypotheses of ecosystem physiology, aboveground-belowground response to a changing climate, and the turnover times of seasonally dynamic carbon pools (Epron et al., 2012), studies that were previously limited to laboratory studies (e.g. Schnyder et al., 2003; Lehmeier et al., 2008, 2010a, b) or inferred from annual changes in biomass measured in the field. Recent findings have illustrated the complexity of dynamic processes that interact at the ecosystem scale. This calls into question the applicability of simple two-end member mixing models in complex systems with multiple sources (Kayler et al., 2010a) and poses a significant challenge for ecosystem studies, as outlined below.

Heterogeneous flux sources

Ecosystem respiration is a complex mixture of isofluxes from a range of biotic and abiotic sources that span the soil to vegetation canopy continuum (see Badeck et al., 2005 and Bowling et al., 2008 and literature therein). These sources contribute with distinct isotopic signatures at time scales from daily (Bowling et al., 2003; Mortazavi et al., 2006; Werner et al., 2006; Kodama et al., 2008; Unger et al., 2010a; Wingate et al., 2010a) to seasonal cycles (Griffis et al., 2004; McDowell et al., 2004; Ponton et al., 2006; Alstad et al., 2007; Schaeffer et al., 2008). These are based partly on phenology and disturbance regimes and all exhibit different effects on component fluxes. The challenge to advance our understanding of δ\(^{13}\)C\(_R\) lies in identifying and quantifying these fluxes and isotopic signatures of important ecosystem components (e.g. Unger et al., 2010a; Epron et al., 2011; Barbour et al., 2011a). This is especially important to test hypotheses about temporal δ\(^{13}\)C\(_R\) patterns, for example, if δ\(^{13}\)C\(_R\) dynamics are heavily influenced by a sole component flux, resulting in a poorly mixed ecosystem source signal. Similarly, species-specific transport times of recent assimilates (Epron et al., 2011) can potentially delay the photosynthetic response signal in δ\(^{13}\)C\(_R\), or abiotic phenomena (following section) can obscure component iso-fluxes (e.g. Ekblad et al., 2005; Knohl et al., 2005). In these cases, deciphering the drivers behind δ\(^{13}\)C\(_R\) may become increasingly difficult.

Abiotic influences

Analyses of δ\(^{13}\)C\(_R\) may lead to the identification of drivers and mechanisms underlying the dynamics of ecosystem metabolism; yet, other abiotic processes that are also affected by biological drivers (e.g. temperature) may amplify, dampen or time-lag responses in δ\(^{13}\)C\(_R\), obfuscating the signal of biological respiration (Brüggemann et al., 2011). Soil respiration, which can represent 20 to 70% of total ecosystem respiration, is an integrative signal driven by many abiotic and biological processes. Recent studies have shown that factors such as diffusivity of soil CO\(_2\), dissolution of CO\(_2\) from bicarbonates, and advection of soil gas may be responsible for strong 1\(^{3}\)-isotopic disequilibria between the CO\(_2\) efflux at the soil surface and concurrent soil respiration (Crow et al., 2006; Kayler et al., 2008, 2010a; Nickerson and Risk, 2009; Ohlsson, 2009; Gannitzer et al., 2011). Likewise, the oxygen isotope composition of soil respired CO\(_2\) (δ\(^{18}\)O\(_2\)) not only carries the isotopic signature of the soil water it interacted with, but also is influenced by the carbonic anhydrase in soil microorganisms that accelerate isotopic equilibration between CO\(_2\) and soil water (Wingate et al., 2009, 2010b). Despite their potential to propagate uncertainties in isotopic information through the soil–canopy continuum, such processes merit inclusion in isotope ecosystem models, enhancing the interpretation of patterns and drivers of δ\(^{13}\)C\(_R\).

Flux partitioning

Conventional partitioning methods based on eddy covariance methods typically require several days or weeks of data to cover key phenological periods in order to obtain robust regression parameters (e.g. Reichstein et al., 2005), neglecting ecosystem responses at shorter time scales. These are, for example, “switches” of ecosystem states (Baldocchi et al., 2006; Lee et al., 2007) or the pulse-like response of soil respiration to strong rain events, occurring at time scales from minutes to hours (e.g. Xu et al., 2004; Unger et al., 2010b, 2012). It would be helpful if the partitioning scheme could resolve episodic responses of this kind, because it is the transient, non-equilibrium responses that provide a rigorous test of model performance and validity. Assimilating continuous measurements of CO\(_2\) and H\(_2\)O fluxes and their isotopic compositions (e.g. δ\(^{13}\)C, δ\(^{18}\)O, δ\(^{2}\)H) into process-based models should therefore provide a better-constrained solution. Similarly, assimilating chamber-based flux measurements of these isotopic fluxes should help to explain and constrain our model predictions during metabolic switches, especially
when photosynthetic products may become limiting such as during drought (Unger et al., 2010a), rainy periods (Wingate et al., 2010a) or when post-photosynthetic fractionation processes dominate the isofluxes, e.g. at dawn (Barbour et al., 2011a).

2.5 Regional scale isotope variation in precipitation and linkages to carbon cycling

High frequency, spatially dense precipitation isoscapes (i.e. spatial distribution maps of isotope records) over long time periods are continuing to assist our understanding of plant water relations, water sources and the extent to which they are driven by seasonally varying water sources and how these sources vary at the regional, inter-annual to inter-decadal scales (Rozanski et al., 1993; Welker, 2000; Vachon et al., 2007). Knowledge on the spatial distribution of the isotope signatures of the source water taken up by plants is also prerequisite to disentangle the climatic and physiological information laid down as $^{18}$O signal in plant organic matter and isotopic archives (Augusti and Schleucher, 2007) on larger scales. At the regional scale, we now fully appreciate that seasonally snow covered systems provide meltwater to soils and river systems that reflect the highly depleted values of winter precipitation (Dutton et al., 2005; Vachon et al., 2010), and that this snow meltwater allows high rates of stomatal conductance and high rates of carbon fixation (Alstad et al., 1999). The duration and extent to which snowmelt and summer precipitation sources are available to the vegetation may be critical to supporting higher plant water use, thus affecting stomatal conductance as well as carbon fixation and gross ecosystem production. The complexity of seasonal patterns of snow meltwater availability at the regional scale is reflected in the vegetation at higher latitudes where Arctic and North Atlantic Oscillation phase changes are recorded in the carbon and oxygen isotope composition (Welker et al., 2005).

Our emerging understanding of the temporal patterns of $^{18}$O and $\delta$D during swings in the major climate oscillations provides a modern basis for calibrating storm track, climate oscillations and the source water of vegetation in conjunction with carbon fixation rates and variability.

Progress and challenges

Tracing climate phase variation

Understanding the role of moisture sourced from multiple regions (i.e. different storm tracks), and how those sources vary with climate phases (i.e. climate oscillations and modes, such as El Niño) as it affects vegetation carbon fixation is unknown (Holmgren et al., 2001; Birks and Edwards, 2009; Sjostrom and Welker, 2009). We continue to recognize that tree rings may be recorders of the general isotopic history of source water (Briffa, 2000; Csank et al., 2011) regardless of geologic time period. However, understanding the extent to which these moisture sources and climate phases are recorded and how plant physiology alters the source water signal in the long-term growth record of trees is one of the great challenges today.

Isotope tracers in back trajectory analysis

Back trajectory analysis of weather and thus precipitation (Draxler and Hess, 2004; Sjostrom and Welker, 2009) is a modelling tool that has been used extensively to quantify long-distance transport of pollutants, and more recently for studies of isotopic characteristics of precipitation (Burnett et al., 2004). Combining this tool with isotopic measurements of continental precipitation and water vapour (e.g. networks such as MIBA and GNIP) and carbon and water fluxes (e.g. networks such as Fluxnet) may be means by which almost real-time linkages between climate phases, moisture sources, plant water relations, carbon exchange and continental carbon cycling may be possible.

2.6 Isotopic archives and relevant aspects of spatio-temporal integration

Over the past decades, the use of stable isotope ratios in a wide range of materials – from tree, sediment and ice cores to corals, hair, cactus spines, the baleen of whales and fish odoliths – has provided some of the most important and novel insights into the patterns of past environmental changes and organismal response to these changes of almost any type of recorder (Dawson and Siegwolf, 2007). Such archives not only provide a way to look back in time but more recent examples show that one can also assign causes to responses to environmental changes on a mechanistic basis (e.g. Ogée et al., 2009). Stable isotope analysis of biological or abiotic archives can thus provide excellent tracers for spatial- and temporal-integration over different scales. Here we discuss progress and challenges of a few selected examples of biological archives.

Progress and challenges

Isotopic archives in trees

Tree rings enable retrospective analyses of intra- and interannual variation of carbon and oxygen isotope composition and the related ecophysiological drivers over many centuries (Sidorova et al., 2009; Nock et al., 2010; Knorre et al., 2010; Andreu-Hayles et al., 2011; Peñuelas et al., 2011). The advantages of tree rings are that they (i) can be reliably dated with a high temporal and spatial resolution; (ii) contain several proxies (stable C, H, O and N isotopes, tree ring width and tree ring density) in the same matrix (tree ring wood/cellulose), which was formed at the same time, location, and environmental conditions; and (iii) mostly the inclusion of a limited number of trees and species may provide a strong signal. However, single tree ring chronologies provide

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only limited spatial and community integration and report only local signals (ca. $10^{-1}$ to $10^{2}$ m). Signal strength is further reduced by species-specific responses to environmental impacts. Furthermore, the tree response is strongly affected by ontogeny (e.g. Monserud and Marshall, 2001) and site specific properties such as competition, soil type, water and nutrient availability, resulting in a considerable variability of the signal expression, even within the same species (Saurer et al., 1997). Thus, constructions of ecosystem chronologies depend on the combination of several tree ring records from trees of different locations within the same site. This requires additional information, such as knowledge of past species dynamics.

**Isotopic archives of herbaceous vegetation**

The life span of herbaceous vegetation is much shorter than that of trees. However, isotopic reconstructions of climate change in herbaceous vegetation (crops and grassland) are possible if plants were sampled and preserved during the epoch. Such archives are relatively rare and are mainly represented by herbaria (e.g. Penuelas and Azcón-Bieto, 1992). In general, herbarium specimens have been sampled at different locations, so that long-term isotopic records from these involve a spatially disperse representation of a species’ changing isotopic composition. Because of site differences, such isotopic records display relatively high variation. Rare opportunities for community-scale isotopic reconstructions are presented by long-term (agro-) ecological experiments with crops and grassland where biomass samples have been stored in dedicated archives (Zhao et al., 2001; Köhler et al., 2010).

**Grazer tissues as isotopic archives**

For grassland, an analogy to tree rings is given by the yearly rings (annuli) of horns (or hoofs) of obligate grazers (Barbosa et al., 2009, 2010). These can yield carbon isotopic records over many years, which reflect that of grassland vegetation (Schnyder et al., 2006). The spatial integrations of tree and horn ring isotopic compositions are quite contrasting: local and stationary for the tree, and vast and cyclic for horns, reflecting visits of the different parts of the year-round grazing grounds. Still, the use of grazer tissue for reconstructions of grassland isotopic chronologies usually rests on a number of assumptions, e.g. concerning the selectivity of grazing, the constancy of the relationships between isotopic composition of grazer tissues, and contributions of diet components of differential digestibility (Wittmer et al., 2010). Such assumptions can be and should be validated (Wittmer et al., 2010). A significant advantage of keratinous tissue (horn, hair/wool and hoofs) is given by its homogenous chemical composition, which reduces variation associated with metabolic isotope fractionation that can be significant in chemically heterogeneous tissue.

**Micro-scale environmental record**

A particular case of small-scale environmental records are carbon and oxygen isotope ratios of non-vascular plants (NVP). Cyanobacteria, algae, lichens, and bryophytes integrate local changes of CO$_2$ (e.g. Máguas and Brugnoli, 1996) and water over long time periods due to their passive exchange with environmental conditions, low growth rates (ca. 0.02–30 mm a$^{-1}$) and long life spans (hundreds to thousands of years). Therefore, NVP can be used, for example, for geochronologic aging (e.g. lichenometry), particularly dating deposited surfaces over the past 500 years with an accuracy of 10% error (Armstrong 2004). The $\delta^{13}$C of NVP archives environmental impacts over the whole life span in bulk organic material, and over several years if a chronosequence is sampled from the thallus margins or young shoots. Short-term and online records can be obtained from analysing respired CO$_2$ and extracted bulk water. $\delta^{13}$C of NVP can be used to trace environmental CO$_2$ gradients (Flanagan et al., 1999; Lakatos et al., 2007; Meyer et al., 2008), whereas fossil bryophytes record ancient CO$_2$ levels (Fletcher et al., 2005, 2006). Additionally, epiphytic plants function as atmospheric water traps (Helliker and Griffiths, 2007; Helliker 2011). Because epiphytic NVP are commonly in equilibrium with water vapour, it is inferred that $\delta^{18}$O of bulk water and organic material might serve as a short and long-term recorder for atmospheric vapour, respectively (Helliker and Griffiths, 2007; Hartard et al., 2008, 2009). In the same line, peat mosses serve as proxies for palaeoenvironmental changes (Loader et al., 2007; Lamentowicz et al., 2008; Moschen et al., 2009; Loisel et al., 2010). However, approaches that use oxygen isotopes as long-term recorder of environmental conditions need to account for the contributions of the different water signals from rain, dew and vapour, as well as physiological offsets which add considerable uncertainties (Moschen et al., 2009).

**3 New technical and methodological developments in stable isotope research**

The past decade has seen tremendous progress in the development of new techniques that complement or rival traditional Isotope Ratio Mass Spectrometry (IRMS) for the determination of stable isotope abundances. This has lead to new dimensions in measurement speed, number of quantifiable isotopologues and sensitivity, increased the repeatability, precision and sample turn-over considerably, and offered new opportunities for in situ observations at natural abundance and in tracer experiments. Most important for carbon and water cycle research was the introduction of instruments using light absorption properties of small molecules for determination of stable isotope abundances, as well as the introduction of innovative techniques for compound-specific sample extraction.
Laser absorption spectroscopy (LAS)

The development of absorption spectroscopy instrumentation (LAS) provided new dimensions of measurement speed and number of quantifiable isotopologues offering data richness that had never been possible to achieve in field-deployable instrumentation (e.g. Bowling et al., 2003, Kammer et al., 2011, Sturm et al., 2012; but see Schnyder et al., 2004). The laser absorption spectroscopy is based on analysis of absorption of light in selected wavelengths in the near and mid-infrared to determine the mole fractions of individual isotopologues (Kerstel, 2004; Kerstel and Gianfrani, 2008; Fried and Richter, 2006). Optical measurement methods based on Fourier Transform Infrared Spectroscopy (FTIR), Cavity Ringdown Spectroscopy (CRDS), Integrated Cavity Output Spectroscopy (ICOS) and Tunable Diode Laser Absorption Spectroscopy (TDLAS) now approach levels of detection of small-molecule isotopologues comparable to laboratory-based isotope ratio mass spectrometers (IRMS). Measurement by absorption spectroscopy is non-destructive and can therefore be repeated to increase measurement precision (Werle et al., 2004). Furthermore, LAS enables a high temporal resolution of accurate isotope ratios, an ideal property for the visualisation of processes and temporal variability (e.g. Bowling et al., 2003; Lee et al., 2005; Tuzson, 2008). Further, new multi-species instruments that are becoming available enable so-called “clumped isotope” measurements (Eiler, 2007), wherein the occurrence of two heavy isotopes in the same molecule can serve as a unique stable isotope tracer itself.

Compound Specific Isotope Analysis (CSIA)

The IRMS has experienced technological and methodological development, particularly Compound Specific Isotope Analysis (CSIA), which includes IRMS coupled to Gas Chromatography-Combustion (GC-C-IRMS; Maier-Augenstein, 1999) or Liquid Chromatography (LC-IRMS; Godin et al., 2007). This facilitates the analysis of different compounds such as structural and labile carbohydrates extracted from plant organs, leaf wax alkanes, phloem sap and soil fractions (see review by Sachse et al., 2012). For compound specific isotope analysis, the extraction method is crucial and might strongly affect the results obtained (Richter et al., 2009). Moreover, the need for derivatization of polar metabolites for GC-MS (gas chromatography-mass spectrometry) analysis and thus the introduction of additional carbon and oxygen via the derivatization agent to the analyte complicates the measurement of natural abundance stable isotope composition of these compound classes (e.g. Gross and Glaser, 2004). This problem does not occur with LC-IRMS systems, which are currently, however, restricted to carbon isotope analyses.

Nuclear Magnetic Resonance (NMR)

At the advent of development of new techniques for nuclear magnetic resonance spectroscopy (NMR), new options arise for studies of, e.g. starch-sugar partitioning and complementary information on (photo-)respiration by analyses of non-homogeneous distribution of $^{13}$C within carbohydrate molecules (e.g. Gilbert et al., 2009, 2011). Analogously, options to distinguish between different water pools within the plant arise from new techniques for $^{18}$O positional analyses by novel derivatiation approaches (Sternberg et al., 2006).

Nano-scale secondary ion mass spectrometers (NanoSIMS)

Linking isotopic analysis with high resolution microscopy has provided significant progress of spatially resolved information on the molecular and isotopic compositions of (biological) materials. New Nano-scale Secondary Ion Mass Spectrometers (NanoSIMS) represent a significant improvement in sensitivity and spatial resolution (down to 50 nm). In a destructive manner, NanoSIMS analysis involves continuous bombardment of the sample surface with an ion beam and subsequent analysis of the released secondary ions according to their mass-to-charge ratios (Herrmann et al., 2007). Although adequate sample preparation remains challenging, imaging mass spectrometry via NanoSIMS represents a promising avenue for mapping the spatial organisation, metabolic pathways and resource fluxes within cells, plants and at the root–fungus–soil interface, in particular in labelling studies (e.g. Clode et al., 2009).

Progress and challenges

The pool of various new and improved techniques currently available for application of stable isotopes in environmental, physiological and ecological research is large. However, from the user's perspective, particularly for laser absorption spectroscopy, some general issues, as described below, should be resolved.

Instrument accuracy and calibration

Calibration biases for water vapour isotope laser spectrometers can result, for instance, from evaporation efficiency of the reference water, instrument nonlinearity and impurity of the carrier gas. Calibration of water vapour analysers is done, for example, using a liquid water injector (“dripper”) into a flow of dry air (Lee et al., 2005; Wen et al., 2008; Baker and Griffis, 2010; Griffis et al., 2010; Sturm and Knohl, 2010). In addition, a heated vaporisation system is used wherein the liquid standard is completely vaporized without fractionation. Nevertheless, any concentration dependence in the analyser itself can bias the overall calibration of the instrument, especially when measuring ambient water vapour of widely varying mixing ratios (Lee et al., 2005; Wen et al., 2008;
Schmidt et al., 2010; Sturm and Knohl, 2010). For CO2, calibration against two or more mixtures of CO2 and dry air, which are tied to international reference standards, are critical. Impurities in the water sample to be analysed can cause a spectral interference with organic contaminants and have been observed in analysis of liquid samples extracted from biological sources, e.g. leaf water (West et al., 2010; Schultz et al., 2011).

High instrument precision at short detection intervals

Free of sample preparation and processing, new optical techniques can achieve much faster detection than IRMS. In-situ measurements of CO2 and H2O isotope ratios in ambient air, especially if made on a long-term basis and calibrated precisely, can provide a powerful tool for atmospheric inverse analysis of the terrestrial carbon sink and tracking of water transport in the atmosphere. However, to measure the source/sink signature properly near the land surface, one should interface the isotopic analyser with plant (Bour et al., 2007; Barthel et al., 2011) and soil chambers (e.g. Wingate et al., 2010a, b) and deploy it in the gradient-diffusion mode either over the vegetation (Griffis et al., 2004) or over the soil surface inside the canopy (Santos et al., 2010), or combine it with a sonic anemometer for direct eddy covariance measurement of isotopic fluxes (Lee et al., 2005; Griffis et al., 2008, 2010) or landscape scale measurements in high elevation or airborne conditions (e.g. Tuszon et al., 2010). In all these configurations, suitable interfaces between the analyser and the sample and calibration periphery are useful. The system as a whole must be robust and designed and tuned for minimal interference, memory effects or signal drifts. Fast detection is particularly critical for eddy covariance applications, which require an instrument response to be faster than 10 Hz and relies on continuous-flow sampling. However, fast detection is also desired for chamber based measurements in studies of short-term events, such as water vapour and CO2 flux pulses after rain (Santos et al., 2010; Unger et al., 2012). Maximizing precision at short integration times and maintaining accuracy for long periods should be a high priority in future instrument development.

Instrument and infrastructure cost

High instrument and maintenance costs limit the broad adoption of new technologies in field research. It is highly desirable that the costs are brought down to a level comparable to that of a broadband infrared gas analyser, which is now an indispensable tool for ecosystem carbon and water flux monitoring worldwide (Baldocchi et al., 2001). We envision the development of a network with real-time observations of isotopic fluxes of CO2 and H2O to help diagnose changes in biospheric processes. This can become a realistic goal if instrumentation costs are lower.

Multi-isotopologue instruments

New instruments are becoming available and enable so-called “clumped isotope” measurements (Eiler, 2007), wherein the occurrence of two heavy isotopes in the same molecule can serve as a unique stable isotope tracer itself. Currently its applicability as a paleo-thermometer is being tested. The basic assumption is based on the observation that the heavier molecules and atoms are not randomly distributed within the same matrix, but rather form a clumped aggregate of substrates with the heavier isotope. For the distinction of such clumped isotopes highly sensitive instruments are needed.

4 Outlook

New research opportunities at all scales of isotope biogeochemistry of carbon and water are arising from deepened process-based understanding and improved analysis tools, together with the development of mechanistic models. Especially combinations of multiple isotope and non-isotope variables have the potential to stimulate our understanding across a wide range of scales, including leaves, plants, ecosystems, and the atmosphere. The scale-spanning assessment of carbon and water fluxes is, on the one hand, a great opportunity offered by stable isotope approaches. On the other hand, deeper insights into the multitude of processes affecting carbon and oxygen isotope discrimination during photosynthesis and transpiration, as well as during downstream metabolic processes, are challenging a generalisation of the information contained in the isotopic signature and a transfer to higher temporal and/or spatial scale. One example is the knowledge that plant phenology or growth patterns (Sect. 2.1.3) might complicate the comparison of the isotopic composition of bulk material between species. However, we can apply appropriate techniques such as the assessment of organic matter pools with a well defined turnover time and chemical composition to avoid misinterpretation. Moreover, experimental designs focussing on changes in environmental conditions or species interactions and on the effect of such changes on the isotopic composition can often overcome the problem. While the isotopic composition might not be directly comparable between species, the direction and magnitude of change can give quantitative information on physiological reactions within and between species, communities and ecosystems.

At the leaf-level (see Sect. 2.1), combined analyses of different isotopes might lead to a better understanding of mesophyll conductance and related components, including diffusion through intercellular airspaces and transport through barriers in cells such as the cell wall, membranes, or stroma. It might also help to assess the possible role of coenzymes (membrane proteins acting as pores for CO2) in facilitating and controlling transport of CO2. Combined measurements
of the isotopologues of CO$_2$ and H$_2$O will further allow quantifying the extent of equilibration between dissolved CO$_2$ and leaf water, and thus can provide a non-invasive reconstruction of leaf water dynamics. These are critical aspects for validation and further development of carbon and water isotope approaches and models. Information on different species and ecotypes will in turn enhance our understanding of the different morpho-physiological factors controlling carbon and water fluxes and, hence, water use efficiency of leaves.

Although the last ten years have seen a large increase in knowledge of post-carboxylation fractionation phenomena (see Sect. 2.1.2), we expect no slowdown in the development of this field. In part, empirical progress will be facilitated by improvements in NMR technology as well as in derivatisation techniques (see Sect. 3) which permit measurements of natural intramolecular isotope distribution patterns in intermediates of primary and secondary metabolism, and respiratory substrates. Dynamic labelling experiments with $^{13}$C-enriched or depleted CO$_2$ or with (intra-molecular) position-labelled metabolites will permit better assessment of metabolic networks and turnover times of different carbon pools. Such work will also enhance our understanding for the metabolic causes of variations in post-carboxylation fractionation. Temporal dynamics of apparent fractionation during dark respiration may vary, depending on the identity of the different metabolic intermediates, their synthesis pathways and metabolic functions as well as on the demand for substrates in the respiratory pathways.

Investigations of natural intra-molecular $^{13}$C and $^{18}$O distribution patterns might also be key to quantify isotope fractionation phenomena during loading, phloem transport and unloading of different organic compounds (see Sect. 2.2). These include assessments of isotopic exchange reactions along the path from leaves to sites of assimilate use, and fractionation or isotopic exchange during biosynthetic processes such as cellulose synthesis. Such approaches may elucidate the mechanisms underlying spatio-temporal variation of $\delta^{13}$C and $\delta^{18}$O during transfer from the chloroplast to heterotrophic tissues, the rhizosphere/soil and atmosphere. The mechanistic understanding, on the other hand, will strengthen climatological and physiological interpretation of tree ring cellulose and similar isotope archives such as grass, sediments, hair, horn, or tooth enamel of herbivores (see Sect. 2.6). We are of the strong opinion that a deeper understanding of fractionation phenomena in the downstream metabolism – as expressed in quantitative models – is still in its infancy.

It is, therefore, not surprising that the interpretation of ecosystem scale fractionation remains challenging (see Sect. 2.4). We expect that significant steps for resolving this complexity will include similar approaches as advocated for leaf- and plant-level studies: (i) joint flux measurements of the different isotopologues of CO$_2$ and H$_2$O in natural systems – which will enable a better distinction of the CO$_2$ and H$_2$O flux components and pools, (ii) tracing metabolite and intramolecular labelling patterns between and within system components in artificial setups as well as in field labelling experiments – shedding light on allocation, turnover of different carbon pools as well as plant-soil-atmosphere interaction, and (iii) hypothesis-testing mesocosm-scale experiments – testing our system-scale understanding. Insights from these approaches may then help to improve and test stable-isotope-enabled models of carbon and water fluxes at the ecosystem scale.

Regional-scale studies (see Sect. 2.5) of the water isotope cycle are becoming more important to our understanding of synoptic climates, ecosystem processes, the role of abiotic processes (e.g. temperature of condensation), moisture sources, and storm tracks on the ecohydrology of entire landscapes and continents. However, isotope fractionations are quite uncertain on global and continental scales and it is therefore important to identify robust features that can be constrained by large-scale isotope observations. C$_4$-plant distribution is one such feature that might become well constrained by $^{13}$C isotopes. But isotope studies will benefit greatly from the combination with other non-isotope tracers also on landscape, regional and continental scales. It might be the tapping into the above-mentioned multitude of information that will advance the usage of isotope signals on the global scale.

In conclusion, we are in the midst of a rapid growth in process-based understanding of the behaviour of carbon and oxygen stable isotopes in organisms and in the environment. On the one hand, we are increasingly recognising the complexity of $^{13}$C and $^{18}$O fractionation processes and their spatial and temporal variation. On the other hand, new technologies (see Sect. 3) can deliver high resolution records of short- and long-term variability in isotope signatures, overcoming the constraints of earlier laborious procedures. New analytical tools and process-based understanding will allow further development of isotope-enabled biogeochemical models for investigations of the complex interplay of soil, plant, ecosystem and atmosphere processes in the carbon and water cycles.
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