Chapter 7
Tracing Carbon Fluxes: Resolving Complexity Using Isotopes

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7.1 Introduction

All trophic systems, from a single cell to the global biosphere, depend on photosynthesis and metabolism of reduced carbon substrates. Cells and ecosystems are, in fact, interconnected and interdependent metabolic networks, which are operated by carbon substrate fluxes. Biotic and abiotic stresses can perturb these fluxes at different scales of biological organisation, from cell to organism (Chap. 1). Such perturbations can affect substrate partitioning between biochemical pathways and allocation between parts of an organism, potentially generating/involving tradeoffs between growth and defence activities (Chaps. 5, 10–12). Knowledge of these responses to stress can enhance our understanding of the controls and mechanisms of carbon fluxes in plants, plant–microbe associations and ecosystems. Moreover, it provides a mechanistic foundation for physiologically based models of plant growth and functioning (Chaps. 15, 17 and 18). At the organism level, the mechanisms underlying carbon cycling include metabolic pathways, transport processes, deposition and mobilisation of stores, synthesis of structural compounds, and environmental and genetic effects on these mechanisms. At the larger scale, such mechanisms may concern the structure and operation of trophic networks or the stability of ecosystems.

Isotope methodologies are useful tools for tracing carbon substrate fluxes, in autotrophic and heterotrophic organisms and trophic networks in ecosystems. A large diversity of (artificial and natural) tracer approaches is available for such investigations. These include feeding of position-labelled ^{13}C substrates; pulse- or

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R. Matyssek et al. (eds.), *Growth and Defence in Plants*, Ecological Studies 220,
DOI 10.1007/978-3-642-30645-7_7,

dynamic labelling with the radio-active short-lived ^{11}C and long-lived ^{14}C or the stable ^{13}C in CO_2; and tracing of the natural alteration of isotopic signals, in photosynthetic reactions and (post-photosynthetic) metabolic pathways. As we show below, there are specific isotope approaches to address questions on very different temporal and spatial scales. For instance the metabolic fluxes ("fluxome") in heterotrophic cells are usually analysed with position-labelled substrates (with high artificial enrichment of the rare isotope), whereas regional- and global-scale carbon fluxes are mostly traced on the basis of natural ^{13}C or ^{14}C signals.

Approach-specific mathematical tools are used to analyse the mechanisms underlying tracer time courses in organisms and ecosystems. In this chapter, we discuss general principles of different carbon isotope tracer methodologies and the specifics of their use in studies of processes at various time frames and scales of biological complexity. Then, we illustrate how the analytical tool "compartmental modelling" can help to analyse tracer time courses. In particular, we demonstrate how compartmental modelling can be used to (1) assess the relative merits of pulse- and dynamic (continuous) labelling for the quantification of carbon pools and fluxes, (2) constrain hypotheses of the topology (architecture, structure) of metabolic systems and (3) elucidate the effect of fructan turnover on the half-lives of fructose, glucose and sucrose in grass leaves. We point out constraints associated with scales of application of different approaches. Finally, we advocate the joint use of different isotope methodologies in future work.

7.2 Principles of Isotopic Tracer Methodologies

"Tracing carbon fluxes" means tracking carbon atoms in chemical reactions or during displacement. Isotopes are ideally suited for this purpose. The word "isotope" derives from the Greek words *isos* and *topos*, which refer to occupation of the "same place" in the periodic table of elements. The isotopes of an element differ in mass, because of a different number of neutrons, but they undergo the same chemical reactions and physical processes. Since they behave the same, alteration of the isotopic composition of a substrate does not (or only minimally) disturb the metabolic and transport pathways. Rather it provides an identifiable tag, or label, with which the course of a biological process can be traced or tracked without disturbance. Detection methods include mass spectrometry, spectroscopy, nuclear magnetic resonance, or radioactive decay measurements (De Groot 2004, 2008). Isotope techniques were proved useful to partition photosynthesis and respiration and to quantify carbon allocation to different compartments and partitioning into different biochemical compounds at various scales, from the cell to the globe (e.g. Bassham et al. 1950; Ludwig and Canvin 1971; Ryle et al. 1976; Geiger and Fondy 1979; Kouchi and Yoneyama 1984; Thorpe and Minchin 1991; Ciais et al. 1995; Yakir and Wang 1996; Schimel 1995; Fung et al. 1997; Gebbing et al. 1998; Gebbing and Schnyder 1999; Randerson et al. 1999; Hanson et al. 2000; Schnyder et al. 2003; Schnyder and Lattanzi 2005; Heinemeyer et al. 2006; Grimoldi et al. 2006; Tcherkez et al. 2009; Gamnitzer et al. 2009; Grams et al. 2011).

There are two principal ways by which isotopes can produce traceable signals in study objects. Either the signal is created artificially, by exposure to isotopically altered substrate, or it arises naturally in metabolism or transport processes. Artificial tracer approaches have made use of the radioactive short-lived ^{11}C (half-life 20.5 min) and long-lived ^{14}C (5,760 years) as well as the stable ^{13}C. Methods of label provision include exposure to isotopically altered CO_2 (Ludwig and Canvin 1971; Geiger 1980; Leavitt et al. 1994; Loreto et al. 1999; Haupt-Herting et al. 2001; Deléens et al. 1983; Gamnitzer et al. 2009) or feeding with uniformly or position-labelled organic substrates, such as sugars and amino acids (Libourel and Shachar-Hill 2008; Schwender 2009; Kruger and Ratcliffe 2009). In the latter, the intra-molecular labelling pattern, at metabolic and isotopic steady state, reflects the label redistribution in metabolic networks and, hence, the metabolic fluxes in the system.

Natural isotope signals are due to different reaction speeds of distinct isotopes in various biochemical and physical processes. These cause isotope fractionation (discrimination) in biochemical and physical processes in photosynthesis and metabolism (Deines 1980; Farquhar et al. 1989; Ehleringer et al. 2000; Ghashghaie et al. 2003; Hobbie and Werner 2004; Tcherkez and Farquhar 2005; Tcherkez and Hodges 2008). Thus, primary CO_2-fixation mechanisms (C3, C4 and marine systems) generate distinct isotopic signals (Bender 1971; O'Leary 1981; Farquhar et al. 1989). Furthermore, the isotope effect on pyruvate-dehydrogenase causes a depletion of ^{13}C in the metabolites of acetyl-CoA and lipids (DeNiro and Epstein 1977; Melzer and Schmidt 1987). Also, the fructose-producing aldolase reaction of the chloroplast prefers ^{13}C, which causes a ^{13}C-enrichment of leaf starch stored during photosynthesis (Gleixner and Schmidt 1997). Thus, sucrose produced from the remaining triose phosphates ("day sucrose") is ^{13}C-depleted, whereas that synthesised at night from depolymerised starch is ^{13}C-enriched, since it inherits the ^{13}C signal from starch (Cernusak et al. 2009). Such isotopic signals are useful tracers of metabolism (e.g. Tcherkez et al. 2003). A difficulty in the utilisation of natural isotope signals in primary photosynthate is their non-steadiness. For instance, the ^{13}C signal of phloem sap contents can vary significantly in diurnal cycles (Kodama et al. 2008). Such factors can complicate a quantitative evaluation and analysis of tracer data.

There are two popular methods of applying labelled CO_2 (or other substrates) and monitoring the propagation of the tracer: pulse(-chase)-labelling and dynamic (long-term) labelling. The latter method has also been referred to as "continuous" (Gamnitzer et al. 2009) or "steady-state" labelling (Geiger 1980; Schnyder 1992). However, in "fluxomics" studies the term "steady-state labelling" is used to denote a labelling principle in which the labelled precursor (usually a specific isotopomer of a substance) is supplied continuously at constant enrichment, and intra-molecular labelling patterns are measured when the system is in isotopic and metabolic steady state (Ratcliffe and Shachar-Hill 2006).

In dynamic labelling, the labelled substrate (e.g. CO_2, see Fig. 7.1) is supplied continuously during the time course of the studied process, at constant isotopic composition. The amount of tracer in the substance of interest increases continuously during label application until—eventually—all sources/pathways supplying

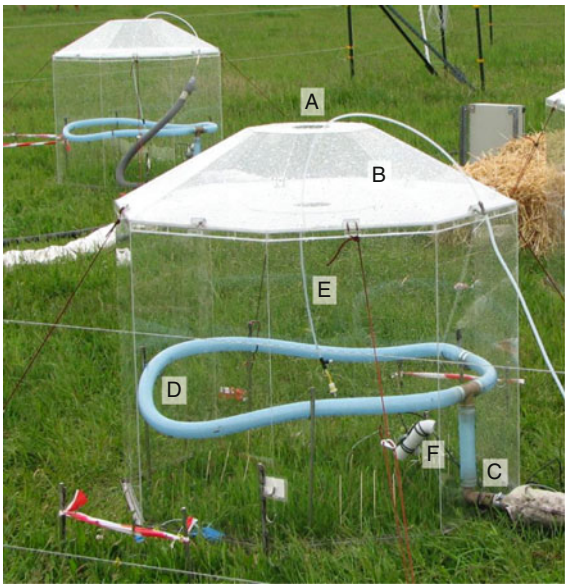


Fig. 7.1 Chamber system for $^{13}CO_2/^{12}CO_2$ labelling of a grassland ecosystem under field conditions (Gamnitzer et al. 2009). An ecosystem section is enclosed in a chamber with an opening in the top (A), therefore named "open-top chamber". A buffer volume (B) prevents ambient air incursion into the chamber headspace. Air with labelled CO_2 (CO_2 with altered $^{13}CO_2$ content) is provided to the chamber via the air supply tube (C) and distributed within the chamber headspace with the perforated tube (D). A sampling tube (E) allows sampling of chamber headspace air for monitoring of labelling conditions inside the chamber and for respiration measurements

the metabolite have reached label saturation (isotopic equilibrium with the labelled substrate). Observations of label content are performed simultaneously with labelling (Geiger 1980; Lattanzi et al. 2005; Lehmeier et al. 2008; Gamnitzer et al. 2009). The change in isotopic composition with time reflects the functional properties of the pool system supplying the synthesis of the metabolite (number and arrangement of pools and the size, turnover rate and contribution of each pool to the synthesis of the metabolite). Compartmental analysis (Atkins 1969; Jacquez 1996) is a useful tool to extract these functional characteristics from labelling kinetics. If the metabolite is not completely labelled, then the metabolite may not have turned over completely, or some unlabelled (or incompletely labelled) source is still contributing to its synthesis. Examples of the latter are metabolites which are synthesised from slowly turning over pools, such as stores or decomposing structural biomass (Lattanzi et al. 2005; Lehmeier et al. 2008).

In pulse-labelling the labelled substrate is provided for a period of time (pulse), which is very short in relation to the time course of the studied process, generally at high isotopic enrichment. Then follows an extended period in which again the unlabelled form of the substrate is provided, as prior to the pulse (chase period) (Austin et al. 1976; Jones et al. 1983; Gregory and Atwell 1991). In such an experiment, the evolution of tracer content in the substance of interest exhibits

two phases: first, it increases as the labelled form of the substrate isotope becomes incorporated, then it decreases as the labelled substance is diluted by incorporation of the unlabelled form of the substrate ("washout" of the tracer). In general, the incorporation of the tracer is not monitored during the pulse-period. Typically, the first observation is made at, or shortly after, the end of the pulse. Further observations occur over the time scale of the process of interest.

Data analysis and interpretation are conducted with approach-specific mathematical tools/simulation models. They include compartmental analysis (Atkins 1969; Jacquez 1996; and see below) and modelling theory and computational methods of metabolic flux analysis (Ratcliffe and Shachar-Hill 2006; Sauer 2006; Schuetz et al. 2007; Libourel and Shachar-Hill 2008; Schwender 2009; Allen et al. 2009) for systems in metabolic steady state. Behaviour of non-steady systems—in natural conditions and usually much larger scales—is analysed with statistical methods such as wavelet coherence analysis (Vargas et al. 2010) or wiggle-matching procedures (Kilian et al. 2000).

Although carbon and its isotopes are the subject of this chapter, we recognise that isotopes of other bio-elements can be useful for carbon metabolism studies. For instance, dual labelling with ^{32}P and ^{14}C ascertained the nature and role of ribulose-1.5-bisphosphate in the reductive pentose phosphate cycle (Benson 1951). As another example, dual labelling experiments with nitrogen and carbon isotopes can help to partition amino-C and carbohydrate-C fluxes (Schnyder and de Visser 1999).

7.3 Processes, Time Frames and Scales of Biological Complexity

The use of carbon isotopes has advanced our understanding of carbon metabolism, allocation and cycling in a great variety of processes: metabolic pathways studies (including uptake/assimilation of CO_2 and biosynthesis of primary, secondary and structural compounds), synthesis and mobilisation of storage compounds, transport across membranes and through vascular conduits, autotrophic and heterotrophic respiration, carbon partitioning in ecosystems, and the roles of different photosynthetic types in the biogeochemistry and biogeography of the earth. These processes have characteristic and distinct time frames. The exchange of carbon in metabolic pathways, such as the Calvin cycle, occurs in minutes (Bassham et al. 1954). Transport of assimilate from leaves to roots in the phloem takes from several minutes to several days, with transport time correlating with plant size. Stores turn over at the scale of a day to many months, and the residence time of carbon in structural biomass varies from months to centuries. Accordingly, the kinetics of label propagation in a system is strongly affected by the types of participating processes.

Moreover, studies of the different processes are performed at different levels of biological organisation and complexity. Metabolic pathway studies (e.g. MFA) are commonly performed in components of cells (chloroplasts, mitochondria, vacuoles

or components thereof), cells or excised tissues. Transport studies require more complex systems, such as whole organs or entire plants. Analyses of sink/source relationships, storage/mobilisation and tissue life span are performed with intact plants or plant stands/communities. On the other hand, carbon residence time was studied on various levels of biological integration from single plant (Lehmeier et al. 2008) to global scale (Bird et al. 1996).

Along with differences in time frames and biological complexity go different challenges in administration/exposure of the label and tracing its fate; metabolic pathway analyses are performed in highly controlled and reproducible conditions and terminated within minutes, whereas the studies of the residence time of carbon in ecosystems generally occur in non-reproducible conditions and require techniques with a resolution of days to centuries.

Also, the experimental methods for tracing label differ between different types of process studies and associated spatial scales (moreover and obviously, there have been transitions in the approaches used over the last 60 years). For instance, the pioneering works of photosynthetic metabolism have used dynamic labelling (*sensu* Ratcliffe and Shachar-Hill 2006) with $^{14}CO_2$ (Bassham et al. 1954), whereas metabolic flux analyses at organelle-, cell- or unicellular organism scales are mainly using steady-state labelling with ^{13}C-position-labelled organic substrates (Libourel and Shachar-Hill 2008) at time scales of seconds to days. Both used high isotopic enrichments. On the other hand, controlled-environment mesocosm studies have employed dynamic labelling with $^{13}CO_2$ at near-natural abundance levels for weeks to months (Deléens et al. 1983; Schnyder 1992). Studies of phloem transport have mainly used CO_2 pulse-labelling with the radioactive short-lived isotope ^{11}C (Minchin and Thorpe 2003) or long-lived ^{14}C (Geiger and Fondy 1979; Geiger 1980).

Studies focusing on long-term processes at ecosystem-level have often used pulse-labelling with $^{13}CO_2$ and $^{14}CO_2$ (Kuzyakov 2006) to investigate the residence time of carbon or the labelling kinetics of respiratory CO_2 (Ostle et al. 2000; Johnson et al. 2002; Carbone and Trumbore 2007; Carbone et al. 2007; Högberg et al. 2008; Bahn et al. 2009). Dynamic labelling experiments in ambient (free air) conditions are methodically challenging, particularly at the ecosystem level (Gamnitzer et al. 2009, 2011). Figure 7.1 shows an example of a field labelling system for weeks-long exposure of a grassland ecosystem to an atmosphere with altered $^{13}CO_2$ content. Yet, free air carbon dioxide enrichment (FACE) experiments have also employed continuous labelling with naturally ^{13}C-depleted CO_2 for CO_2 enrichment (Leavitt et al. 1994). This provides a measurable isotopic label which can be traced in the ecosystem (Glaser et al. 2006; Keel et al. 2006; Bock et al. 2007; Grams et al. 2011; Kuptz et al. 2011). However, the precision and accuracy of labelling (e.g. signal to noise ratio) of FACE systems is generally inferior to that of chamber-based systems (e.g. Gamnitzer et al. 2009).

At much larger scales, such as that of catchments, regions or the globe, the artificial alteration of isotopic content of CO_2 or carbon pools/substrates is generally not feasible. At field scale, C4 crops may be used to trace the fate of carbon in C3 soils (Buchmann and Ehleringer 1998; Bol et al. 2009). At regional and global scale, one must resort to natural isotopic signals, such as the different isotopic

composition of terrestrial and oceanic CO_2 sink, which allow partitioning of land/ocean contributions to the missing global carbon sink (Ciais et al. 1995; Fung et al. 1997; Randerson et al. 1999). However, natural (and bomb) ^{14}C signals provide a powerful tool for studies of soil carbon turnover at decadal to millennial time scales (Trumbore 2006, 2009). A remarkable exceptionality is given by the "anthropogenic" ^{14}C bomb spike that has been used as a tracer (Stenhouse and Baxter 1977) to address research questions from the scale of single organisms to that of the globe (e.g. Broecker et al. 1985; Bird et al. 1996; Richter et al. 1999; Spalding et al. 2008).

7.4 Pulse- *Versus* Dynamic Labelling

The relative merits of pulse- and dynamic labelling were discussed previously (Geiger 1980; Meharg 1994; Kuzyakov 2006; Paterson et al. 2009), but the two approaches were not compared directly and with quantitative methods. Here we quantitatively compare the tracer time courses (the so-called "tracer kinetics") of pulse and dynamic labelling. Based on data from a dynamic labelling experiment, the pool characteristics were determined by compartmental analysis (Fig. 7.2a). The dynamic labelling data suggested that the sink was supplied by a two-pool system, as shown in the inset of Fig. 7.2b. Compartmental analysis revealed that this system was composed of a "metabolic and transport pool" (P_1) with a half-life of 0.1 day, and a "store" with a half-life of 6 days (P_2).

These pool characteristics were then used to derive the tracer kinetics for pulse-chase labelling, based on a 0.8-day-long pulse (Fig. 7.2b). The kinetics of label uptake during the pulse is identical to the initial kinetics of dynamic labelling. The pulse caused a strong labelling of the rapidly turned-over P_1 (63 % label saturation just after the pulse), but a weak labelling of the slowly turned-over P_2 (5 % labelling just after the pulse). In the subsequent washout period, P_1 lost much more label than P_2, as the latter was much less labelled during the pulse. In consequence, detecting the contribution of P_2 in supplying the sink would require adjusting (*i.e.* increasing) the ^{13}C-enrichment of the labelling pulse. Otherwise, pulse-labelling experiments may be "biased" as the contribution of pools with slow turnover goes undetected. For instance, failure to recognise this restriction/disadvantage of pulse-labelling can lead to overestimation of the contribution of current assimilation in supplying a function (sink), and underrating of the role of slowly turned-over stores. Dynamic labelling avoids this problem, provided that the measurement frequency directly after the onset of labelling is high enough to resolve the fast pool(s) and the labelling is continued until (or close to) isotopic saturation of the slow pool(s).

However, there are also advantages for pulse-labelling. For instance, translocation velocity in plants can be assessed simply by providing a short pulse of $^{11}CO_2$ or $^{14}CO_2$ to photosynthesising leaves and monitoring the transit time of the labelled assimilate-pulse using radiation detectors placed at different positions along the translocation path (Geiger and Swanson 1965; Jahnke et al. 1981). Also, fluctuations in allocation patterns in non-steady systems can be detected by

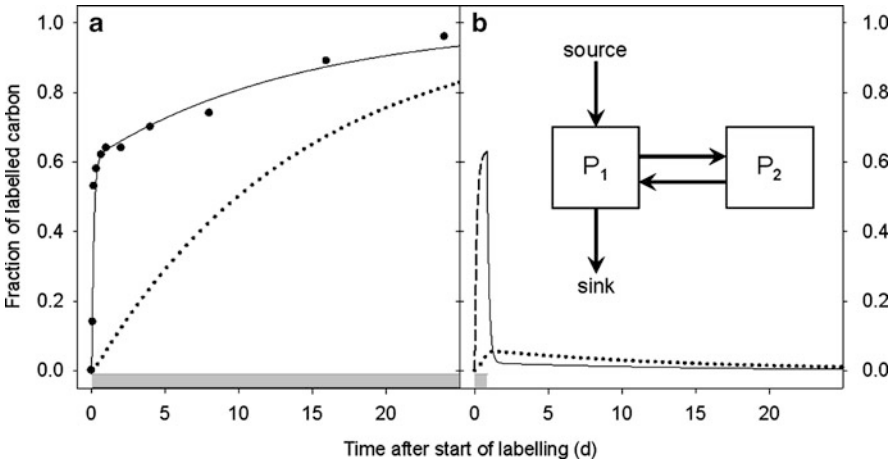


Fig. 7.2 Tracer time course (tracer kinetics) in a "dynamic labelling" (**a**) and a "pulse-chase labelling" experiment (**b**). Tracer kinetics of identical biological systems are compared in (**a**) and (**b**). This system conforms to a two-pool model, shown as an *inset* in (**b**). The system includes a "metabolic and transport pool" (P_1) and a "store" (P_2). Tracer taken up from the source must pass through P_1, before arriving in the sink. But, some of the tracer first cycles through P_2, before being passed on to the sink. In both panels, the labelling duration is indicated by a *grey shaded bar*. The measured data (*filled circles* in (**a**) give the fraction of labelled carbon in the amino-C flux supplying the leaf growth zone of a perennial ryegrass leaf (sink). Plants were grown in a (near-) steady-state in continuous light. The data were obtained from Wild et al. (unpublished) and analysed using procedures as described by Lattanzi et al. (2005). The *continuous line* in (**a**) represents the fit of the two-pool model to the data as obtained with compartmental analysis. In (**b**) the *dashed line* reflects the label increase during the pulse; the *solid line* gives the subsequent decay (washout) kinetics calculated using the same compartmental model as in (**a**), with identical pool characteristics. The *dotted line* in (**a**) and (**b**) give the labelling kinetics of P_2

sequential pulse-labelling of replicates of the system. Furthermore, pulse-labelling studies are experimentally less demanding than dynamic labelling, in particular under field conditions.

7.5 Using Compartmental Modelling to Assess Network Architecture/Topology and Metabolite Compartmentation

One of the present challenges to analysing and understanding metabolic fluxes in plants, as compared with unicellular organisms, is the much greater complexity of plant metabolic networks. To a significant extent, the greater complexity is related to compartmentation, which causes separation of networks, and to the existence and involvement of stores/storage compartments, which are a source of slowly labelled substrate (Kruger et al. 2007; Allen et al. 2009; see above). Failure to consider compartmentation can lead to misinterpretations of labelling patterns (Sweetlove et al. 2008).

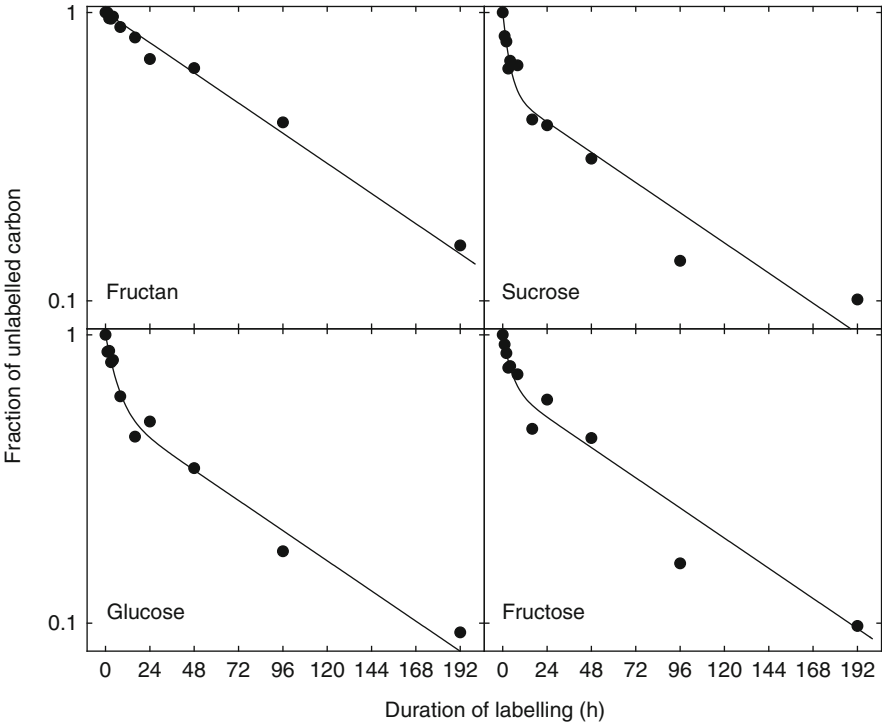


Fig. 7.3 Semi-logarithmic plot of the fraction of unlabelled carbon in fructan, sucrose, glucose and fructose during labelling. The data were obtained from a steady-state $^{13}CO_2/^{12}CO_2$ labelling experiment with *Lolium perenne* grown in continuous light with a high nitrogen supply (unpublished data). Carbohydrates were extracted from the youngest fully expanded leaf of mature tillers. Data modified from Lattanzi et al. (2012)

Compartmental modelling is one of the tools which may assist in resolving some of these problems. It can distinguish distinct pools of a metabolite, if the pools differ in the kinetics of labelling (i.e. slow- versus fast-labelled pools). Such differences are expected for metabolites originating from current assimilation and stores. Here we demonstrate the usefulness of compartmental modelling for this purpose using data from dynamic labelling of the water-soluble carbohydrates (glucose, fructose, sucrose and fructan) in the leaf blades of perennial ryegrass (Fig. 7.3).

The tracer kinetics of fructan, a vacuolar storage carbohydrate, fitted a one-pool (first-order kinetics) model with a half-life of 69 h ($r^2 = 0.98$). Conversely, the tracer kinetics of sucrose, glucose and fructose reflected two-pool systems. Their tracer kinetics fitted double exponential functions of the form $y = a \cdot e^{-b \cdot t} + (1 - a) \cdot e^{-c \cdot t}$. The interpretation of the fit parameters depends on the system structure, which is discussed in detail below. However, to illustrate the power of the compartmental analysis tool, we discuss an example. This is represented by a system in which both pools incorporate and release tracer and in which no exchange occurs between the two pools. In such a system, the parameters a and

$1 - a$ represent the fractional contributions of pools 1 and 2 to the total concentration of the respective carbohydrates. The parameters b and c represent the turnover rates (h^{-1}) of pools 1 and 2, which are directly linked to the respective half-lives.

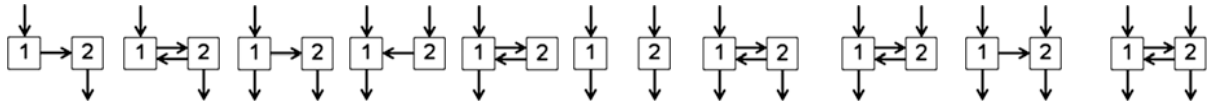
The fast pool (pool 1) of fructose, glucose and sucrose had very similar half-lives: 2.4 h for sucrose ($r^2 = 0.95$), 4.3 h for glucose ($r^2 = 0.98$) and 2.0 h for fructose ($r^2 = 0.96$), consistent with the expectation that they were formed from primary photosynthetic products. A more comprehensive analysis of central carbohydrate metabolism (considering both fructan metabolism and invertase activity) with a four-pool compartmental model demonstrated even faster half-lives of sucrose, glucose and fructose (Lattanzi et al. 2012).

The half-life of the carbon in pool 2 of these carbohydrates was the same as that of the fructan pool. This is consistent with the view that the residence time of carbon in pool 2 of these carbohydrates was controlled by the (vacuolar) fructan pool; the carbon in pools 2 of fructose, glucose and sucrose originated from the turnover of fructan. Fructan degradation yields (mainly) fructose. Part of this is used to form glucose via isomerisation, and both sugars are used for (re-)synthesis of sucrose (Pollock and Cairns 1991). The close similarity of the half-lives of pool 2 of fructose, glucose and sucrose indicates that the metabolic steps leading to sucrose re-synthesis occurred very rapidly. This interpretation was also supported by the low concentrations of pool 2 of fructose and glucose (data not shown). These results demonstrate the usefulness of dynamic labelling and compartmental analysis to unravel differences in the sub-cellular origin of metabolites in complex metabolic networks.

Compartmental modelling can also help to constrain predictions on the topology of networks. This is exemplified by different two-pool models fitted to the data of Fig. 7.2a. Table 7.1 shows the ten variants of two-pool models which differ in structure. One- and three-pool models were also fitted to the data shown in Fig. 7.2a. The one-pool model exhibited a significant lack of fit, whereas three-pool models were not supported by the data due to over-parameterisation (not shown). Among the two-pool models (Table 7.1), model 5 represented a system consisting of a storage compartment which exchanges with a metabolic and transport pool. Biological evidence supported the realism of this model (Wild 2010). The results of the model fits were consistent with the empirical expectation: model 5 fitted the data equally well or better than the other models.

Models 1 and 2 represented a serial arrangement of the two pools, with one pool receiving tracer from the source and the other pool releasing the tracer to the sink end of the system. These models fitted the data very poorly, compared to the other models, suggesting that a serial arrangement of the pools was unlikely. Models 7–10 fitted the data well, but the estimates of pool size and half-life were associated with large errors. These errors were a consequence of over-parameterisation of the models. This means that the models were more complex than was necessary to explain the tracer data. These models were therefore rejected, following the rule of parsimony. Simpler models (models 3–6) fitted the data equally well, but exhibited much less error than models 7–10. Therefore, these simpler models provided the

Table 7.1 Two-pool models fitted to the tracer time course shown in Fig. 7.1A



Parameter	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6	Model 7	Model 8	Model 9	Model 10
Independent parameters	2	3	3	3	3	3	4	4	4	5
r^2	0.49	0.49	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96
F-Value	8.78	3.84	97.84	98.13	97.83	98.12	57.07	57.25	57.25	36.80
$T_{1/2}$ Q_1 (h)	0.0 ± 1.8	11.4 ± 4.7	2.4 ± 0.4	2.4 ± 0.6	2.4 ± 0.4	2.4 ± 0.6	2.4 ± 5.0	2.4 ± 50.2	2.4 ± 2.1	2.4 ± 62.0
$T_{1/2}$ Q_2 (h)	10.1 ± 3.6	0.1 ± 0.0	234 ± 76	234. ± 77	144 ± 53	234 ± 77	198 ± 96,873	219 ± 3,508	234 ± 106	233 ± 1,015,800
Size Q_1	0.1 ± 2.6	16.5 ± 6.8	3.5 ± 0.6	3.5 ± 0.8	5.7 ± 1.2	2.1 ± 0.5	4 ± 505	4 ± 59	2.1 ± 2.2	3 ± 2,975
Size Q_2	14.6 ± 5.2	0.1 ± 0.1	131 ± 52	131 ± 45	129 ± 68	132 ± 43	130 ± 55,499	130 ± 6,307	132 ± 184	131 ± 572,421
p in Q_1	–	–	–	0.61	–	0.61	–	0.66	0.62	0.62
p in Q_2	–	–	–	0.39	–	0.39	–	0.34	0.38	0.38
p out Q_1	–	–	0.61	–	–	0.39	0.72	–	0.61	0.80
p out Q_2	–	–	0.39	–	–	0.61	0.28	–	0.39	0.20

The number of independent parameters is given by the number of adjustable fluxes (which is the total number of fluxes minus 1, as the influx is defined as the unit flux 1 h^{-1}) plus the number of adjustable pool sizes (which is 2) minus the number of constraints due to the steady-state assumption (1 for each pool). The r^2 and F values are the usual statistical information for the best fit of each model to the data. Pool sizes and half-lives ($T_{1/2}$) are given as means +/− SE. Pool sizes are given in relative units, as the import rate (the flux through the system) was assumed to be the unit flux. The parameter p denotes the fraction of the total influx/outflux into/out of pools 1 and 2, respectively. Data from Wild et al. (unpublished)

best reflection of the topology of the system represented in Fig. 7.2a. Among these, models 3 and 6 represented systems serving two sinks, whereas models 4 and 5 served only one. As the experimental system considered here (Fig. 7.2) had only one physical sink, the leaf growth zone, models 3 and 6 seemed unapt. However, it is still possible that this single sink was fed by two distinct metabolic pathways, utilising two (groups of) metabolites with different origins in the supply system. Chemical analysis might reveal this possibility. This example demonstrates that modelling can guide experimentation, by pointing to system features which merit further analysis. Such work could lead to advances in hypothesis development.

Some system properties are sensitive to differences in topology, but others are not. For instance, the half-life estimate of pool 1 was the same for models 3–6. Also, predictions of pool contributions to the total sink flux (or shares of the total source flux) agreed perfectly. So these features were independent of differences in topology, meaning that uncertainties of topology were non-critical for the estimation of these parameters. Conversely, estimates of the half-life of pool 2 and of the size of pools 1 and 2 were dependent on topology, showing that knowledge of model topology is critical for accurate assessment of other system features.

7.6 Conclusions

Today, a wide range of isotope methodologies are available for tracing carbon fluxes at widely differing scales, from cellular metabolic pathways to global biogeochemical cycles. The development of the various methodologies has historic roots in different disciplines of bioscience, and the methods have been used to great advantage in their original disciplines. However, many methods have potential for application outside their traditional discipline. Furthermore, we can expect much benefit from applying different isotope methodologies to the same research questions. Investigations of natural intra-molecular isotope distributions can be combined with ^{13}C-labelling based metabolic flux analysis in microorganisms, plants or plant–microbe associations using paired experimental units for the two approaches. Such joint methodologies could be combined with dynamic labelling and compartmental modelling to shed light on the role of stores/recycling pools in metabolic networks. Inter alia, such work should be performed with plants and plant–microbe associations in non-stressed environments and conditions of abiotic and biotic stress, to further our mechanistic understanding of the real-world controls of tradeoffs in carbon substrate allocation and partitioning in these systems (Chaps. 1 and 20).

References

Allen DK, Libourel IGL, Shachar-Hill Y (2009) Metabolic flux analysis in plants: coping with complexity. Plant Cell Environ 32:1241–1257

Atkins GL (1969) Multicompartment models in biological systems. Methuen, London
Austin RB, Ford MA, Edrich JA (1976) Some effects of leaf posture on photosynthesis any yield in wheat. Ann Appl Biol 83:425–446
Bahn M, Schmitt M, Siegwolf R, Richter A, Brüggemann N (2009) Does photosynthesis affect grassland soil-respired CO_2 and its carbon isotope composition on a diurnal timescale? New Phytol 182:451–460
Bassham JA, Benson AA, Calvin M (1950) The path of carbon in photosynthesis. 8. The role of malic acid. J Biol Chem 185:781–787
Bassham JA, Benson AA, Kay LD, Harris AZ, Wilson AT, Calvin M (1954) The path of carbon in photosynthesis, 21. The cyclic regeneration of carbon dioxide acceptor. J Am Chem Soc 76:1760–1770
Bender MM (1971) Variations in $^{13}C/^{12}C$ ratios of plants in relation to pathway of photosynthetic carbon dioxide fixation. Phytochemistry 10:1239–1244
Benson AA (1951) The identification of Ribulose in $^{14}CO_2$ photosynthesis products. J Am Chem Soc 73:2971–2972
Bird MI, Chivas AR, Head J (1996) A latitudinal gradient in carbon turnover times in forest soils. Nature 381:143–146
Bock M, Glaser B, Millar N (2007) Sequestration and turnover of plant- and microbially derived sugars in a temperate grassland soil during 7 years exposed to elevated atmospheric pCO_2. Glob Change Biol 13:478–490
Bol R, Poirier N, Balesdent J et al (2009) Molecular turnover time of soil organic matter in particle-size fractions of an arable soil. Rapid Commun Mass Spectrom 23:2551–2558
Broecker WS, Peng TH, Ostlund G, Stuiver M (1985) The distribution of bomb radiocarbon in the ocean. J Geophys Res Oceans 90(C4):6953–6970
Buchmann NN, Ehleringer JR (1998) CO_2 concentration profiles, and carbon and oxygen isotopes in C3 and C4 crop canopies. Agric Forest Meteorol 89:45–58
Carbone MS, Trumbore SE (2007) Contribution of new photosynthetic assimilates to respiration by perennial grasses and shrubs: residence times and allocation patterns. New Phytol 176:124–135
Carbone MS, Czimczik CI, McDuffee KE, Trumbore SE (2007) Allocation and residence time of photosynthetic products in a boreal forest using a low-level ^{14}C pulse-chase labeling technique. Glob Change Biol 13:466–477
Cernusak LA, Tcherkez G, Keitel C, Cornwell WK, Santiago LS, Knohl A, Barbour MM, Williams DG, Reich PB, Ellsworth DS, Dawson TE, Griffiths HG, Farquhar GD, Wright IJ (2009) Viewpoint: why are non-photosynthetic tissues generally ^{13}C enriched compared with leaves in C_3 plants? Review and synthesis of current hypotheses. Funct Plant Biol 36: 199–213
Ciais P, Tans PP, White JWC, Trolier M, Francey RJ, Berry JA, Randall DR, Sellers PJ, Collatz JG, Schimel DS (1995) Partitioning of ocean and land uptake of CO_2 as inferred by $\delta^{13}C$ measurements from the NOAA climate monitoring and diagnostics laboratory global air sampling network. J Geophys Res Atmos 100:5051–5070
De Groot PA (ed) (2004) Handbook of stable isotope analytical techniques, vol I. Elsevier, Amsterdam
De Groot PA (ed) (2008) Handbook of stable isotope analytical techniques, vol II. Elsevier, Amsterdam
Deines P (1980) The isotopic composition of reduced organic carbon. In: Fritz P, Fontes JC (eds) Handbook of environmental isotope geochemistry, vol 1. The terrestrial environment A. Elsevier, Amsterdam, pp 329–406
Deléens E, Pavlides D, Queiroz O (1983) Natural ^{13}C abundance as a tracer for the determination of leaf matter turn-over in C3 plants. Physiologie Végétale 21:723–729
DeNiro MJ, Epstein S (1977) Mechanisms of carbon isotope fractionation associated with lipid synthesis. Science 197:261–263

Ehleringer JR, Buchmann N, Flanagan LB (2000) Carbon isotope ratios in belowground carbon cycle processes. Ecol Appl 10:412–422

Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon isotope discrimination and photosynthesis. Annu Rev Plant Physiol Plant Mol Biol 40:503–537

Fung I, Field CB, Berry JA, Thompson MV, Randerson JT, Malmstrom CM, Vitousek PM, Collatz GJ, Sellers PJ, Randall DA, Denning AS, Badeck F, John J (1997) Carbon 13 exchanges between the atmosphere and biosphere. Glob Biogeochem Cycles 11:507–533

Gamnitzer U, Schäufele R, Schnyder H (2009) Observing ^{13}C labelling kinetics in CO_2 respired by a temperate grassland ecosystem. New Phytol 184:376–386

Gamnitzer U, Moyes AB, Bowling DR, Schnyder H (2011) Measuring and modelling the isotopic composition of soil respiration: insights from a grassland tracer experiment. Biogeosciences 8:1333–1350

Gebbing T, Schnyder H (1999) Pre-anthesis reserve utilization for protein and carbohydrate synthesis in grains of wheat. Plant Physiol 121:871–878

Gebbing T, Schnyder H, Kühbauch W (1998) Carbon mobilization in shoot parts and roots of wheat during grain filling: assessment by $^{13}C/^{12}C$ steady-state labelling, growth analysis and balance sheets of reserves. Plant Cell Environ 21:301–313

Geiger DR (1980) Measurement of translocation. Methods Enzymol 69:561–571

Geiger DR, Fondy BR (1979) Methods for continuous measurement of export from a leaf. Plant Physiol 64:361–365

Geiger DR, Swanson CA (1965) Sucrose translocation in sugar beet. Plant Physiol 40:685–690

Ghashghaie J, Badeck FW, Lanigan G, Nogues S, Tcherkez G, Deléens E, Cornic G, Griffiths H (2003) Carbon isotope fractionation during dark respiration and photorespiration in C_3 plants. Phytochem Rev 2:145–161

Glaser B, Millar N, Blum H (2006) Sequestration and turnover of bacterial- and fungal-derived carbon in a temperate grassland soil under long-term elevated atmospheric pCO_2. Glob Change Biol 12:1521–1531

Gleixner G, Schmidt H-L (1997) Carbon isotope effects on the fructose-1,6-bisphosphate aldolase reaction, origin for non-statistical 13C distributions in carbohydrates. J Biol Chem 272:5382–5387

Grams TEE, Werner H, Kuptz D, Ritter W, Fleischmann F, Andersen CP, Matyssek R (2011) A free-air system for long-term stable carbon isotope labeling of adult forest trees. Trees 25:187–198

Gregory PJ, Atwell BJ (1991) The fate of carbon in pulse-labelled crops of barley and wheat. Plant Soil 136:205–213

Grimoldi AA, Kavanova M, Lattanzi FA, Schäufele R, Schnyder H (2006) Arbuscular mycorrhizal colonization on carbon economy in perennial ryegrass: quantification by $^{13}CO_2/^{12}CO_2$ steady-state labelling and gas exchange. New Phytol 172:544–553

Hanson PJ, Edwards NT, Garten CT, Andrews JA (2000) Separating root and soil microbial contributions to soil respiration: a review of methods and observations. Biogeochemistry 48:115–146

Haupt-Herting S, Klug K, Fock HP (2001) A new approach to measure gross CO_2 fluxes in leaves. Gross CO_2 assimilation, photorespiration, and mitochondrial respiration in the light in tomato under drought stress. Plant Physiol 126:388–396

Heinemeyer A, Ineson P, Ostle N, Fitter AH (2006) Respiration of the external mycelium in the arbuscular mycorrhizal symbiosis shows strong dependence on recent photosynthates and acclimation to temperature. New Phytol 171:159–170

Hobbie EA, Werner RA (2004) Intramolecular, compound-specific, and bulk carbon isotope patterns in C3 and C4 plants: a review and synthesis. New Phytol 161:371–385

Högberg P, Högberg MN, Göttlicher SG, Betson NR, Keel SG, Metcalfe DB, Campbell C, Schindlbacher A, Hurry V, Lundmark T, Linder S, Nasholm T (2008) High temporal resolution tracing of photosynthate carbon from the tree canopy to forest soil microorganisms. New Phytol 177:220–228

Jacquez J (1996) Compartmental analysis in biology and medicine. Elsevier, Amsterdam

Jahnke S, Stöcklin G, Willenbrink J (1981) Translocation profiles of ^{11}C-assimilates in the petiole of *Marsilea quadrifolia* L. Planta 153:56–63

Johnson D, Leake JR, Ostle N, Ineson P, Read DJ (2002) In situ $^{13}CO_2$ pulse-labelling of upland grassland demonstrates a rapid pathway of carbon flux from arbuscular mycorrhizal mycelia to the soil. New Phytol 153:327–334

Jones DA, Smith AM, Woolhouse HW (1983) An apparatus for pulse and pulse-chase experiments with $^{14}CO_2$ on attached leaves with known, steady-state rates of photosynthesis. Plant Cell Environ 6:161–166

Keel SG, Siegwolf RTW, Körner C (2006) Canopy CO_2 enrichment permits tracing the fate of recently assimilated carbon in a mature deciduous forest. New Phytol 172:319–329

Kilian MR, van Geel B, van der Plicht J (2000) ^{14}C AMS wiggle matching of raised bog deposits and models of peat accumulation. Quat Sci Rev 19:1011–1033

Kodama N, Barnard RL, Salmon Y, Weston C, Ferrio JP, Holst J, Werner RA, Saurer M, Rennenberg H, Buchmann N, Gessler A (2008) Temporal dynamics of the carbon isotope composition in a *Pinus sylvestris* stand: from newly assimilated organic carbon to respired carbon dioxide. Oecologia 156:737–750

Kouchi H, Yoneyama T (1984) Dynamics of carbon photosynthetically assimilated in nodulated soya bean-plants under steady-state conditions. 1. Development and application of $^{13}CO_2$ assimilation system at a constant ^{13}C abundance. Ann Bot 53:875–882

Kruger NJ, Ratcliffe RG (2009) Insights into plant metabolic networks from steady-state metabolic flux analysis. Biochimie 91:697–702

Kruger NJ, Le Lay P, Ratcliffe RG (2007) Vacuolar compartmentation complicates the steady-state analysis of glucose metabolism and forces reappraisal of sucrose cycling in plants. Phytochemistry 68:2189–2196

Kuptz D, Fleischmann F, Matyssek R, Grams TEE (2011) Seasonal patterns of carbon allocation to respiratory pools in 60-yr-old deciduous (*Fagus sylvatica*) and evergreen (*Picea abies*) trees assessed via whole-tree stable carbon isotope labeling. New Phytol. doi:10.1111/j.1469-8137.2011.03676.x

Kuzyakov Y (2006) Sources of CO_2 efflux from soil and review of partitioning methods. Soil Biol Biochem 38:425–448

Lattanzi FA, Schnyder H, Thornton B (2005) The sources of carbon and nitrogen supplying leaf growth. Assessment of the role of stores with compartmental models. Plant Physiol 137:383–395

Lattanzi FA, Ostler U, Wild M, Morvan-Bertrand A, Decau M-L, Lehmeier CA, Meuriot F, Prud'homme M-P, Schäufele R, Schnyder H (2012) Fluxes in central carbohydrate metabolism of source leaves in a fructan-storing C3 grass – rapid turnover and futile cycling of sucrose in continuous light under contrasted nitrogen nutrition status. J Exp Bot. doi:10.1093/jxb/ers020

Leavitt SW, Paul EA, Kimball BA, Hendrey GR, Mauney JR, Rauschkolb R, Rogers H, Lewin KF, Nagy J, Pinter PJ Jr, Johnson HB (1994) Carbon isotope dynamics of free-air CO_2-enriched cotton and soils. Agric Forest Meteorol 70:87–101

Lehmeier CA, Lattanzi FA, Schäufele R, Wild M, Schnyder H (2008) Root and shoot respiration of perennial ryegrass are supplied by the same substrate pools: assessment by dynamic ^{13}C labeling and compartmental analysis of tracer kinetics. Plant Physiol 148:1148–1158

Libourel IGL, Shachar-Hill Y (2008) Metabolic flux analysis in plants: from intelligent design to rational engineering. Annu Rev Plant Biol 59:625–650

Loreto F, Delfine S, Di Marco G (1999) Estimation of photorespiratory carbon dioxide recycling during photosynthesis. Aust J Plant Physiol 26:733–736

Ludwig LJ, Canvin DT (1971) An open gas-exchange system for simultaneous measurement of CO_2 and $^{14}CO_2$ fluxes from leaves. Can J Bot 49:1299–1313

Meharg AA (1994) A critical review of labeling techniques used to quantify rhizosphere carbon-flow. Plant Soil 166:55–62

Melzer E, Schmidt HL (1987) Carbon isotope effects on the pyruvate-dehydrogenase reaction and their importance for relative carbon-13 depletion in lipids. J Biol Chem 262:8159–8164

Minchin PEH, Thorpe MR (2003) Using the short-lived isotope ^{11}C in mechanistic studies of photosynthate transport. Funct Plant Biol 30:831–841

O'Leary MH (1981) Carbon isotope fractionation in plants. Phytochemistry 20:553–567

Ostle N, Ineson P, Benham D, Sleep D (2000) Carbon assimilation and turnover in grassland vegetation using an in situ $^{13}CO_2$ pulse labelling system. Rapid Commun Mass Spectrom 14:1345–1350

Paterson E, Midwood AJ, Millard P (2009) Through the eye of the needle: a review of isotope approaches to quantify microbial processes mediating soil carbon balance. New Phytol 184:19–33

Pollock CJ, Cairns AJ (1991) Fructan metabolism in grasses and cereals. Annu Rev Plant Physiol Plant Mol Biol 42:77–101

Randerson JT, Thompson MV, Field CB (1999) Linking ^{13}C-based estimates of land and ocean sinks with predictions of carbon storage from CO_2 fertilization of plant growth. Tellus B Chem Phys Meteorol 51:668–678

Ratcliffe RG, Shachar-Hill Y (2006) Measuring multiple fluxes through plant metabolic networks. Plant J 45:490–511

Richter DD, Markewitz D, Trumbore SE, Wells CG (1999) Rapid accumulation and turnover of soil carbon in a re-establishing forest. Nature 400:56–58

Ryle GJA, Cobby JM, Powell CE (1976) Synthetic and maintenance respiratory losses of $^{14}CO_2$ in uniculm barley and maize. Ann Bot 40:571–586

Sauer U (2006) Metabolic networks in motion: ^{13}C-based flux analysis. Mol Syst Biol 2:62

Schimel DS (1995) Terrestrial ecosystems and the carbon-cycle. Glob Change Biol 1:77–91

Schnyder H (1992) Long-term steady-state labelling of wheat plants by use of natural $^{13}CO_2/^{12}CO_2$ mixtures in an open, rapidly turned-over system. Planta 187:128–135

Schnyder H, de Visser R (1999) Fluxes of reserve-derived and currently assimilated carbon and nitrogen in perennial ryegrass recovering from defoliation. The regrowing tiller and its component functionally distinct zones. Plant Physiol 119:1423–1435

Schnyder H, Lattanzi FA (2005) Partitioning respiration of C3-C4 mixed communities using the natural abundance ^{13}C approach – testing assumptions in a controlled environment. Plant Biol 7:592–600

Schnyder H, Schäufele R, Lötscher M, Gebbing T (2003) Disentangling CO_2 fluxes: direct measurements of mesocosm-scale natural abundance $^{13}CO_2/^{12}CO_2$ gas exchange, ^{13}C-discrimination, and labelling of CO_2 exchange flux components in controlled environments. Plant Cell Environ 26:1863–1874

Schuetz R, Kuepfer L, Sauer U (2007) Systematic evaluation of objective functions for predicting intracellular fluxes in Escherichia coli. Mol Syst Biol 3:119

Schwender J (ed) (2009) Plant metabolic networks. Springer, Dordrecht, 331 p

Spalding KL, Arner E, Westermark PO et al (2008) Dynamics of fat cell turnover in humans. Nature 453:783–787

Stenhouse MJ, Baxter MS (1977) Bomb ^{14}C as a biological tracer. Nature 267:828–832

Sweetlove LJ, Fell D, Fernie AR (2008) Getting to grips with the plant metabolic network. Biochem J 409:27–41

Tcherkez G, Farquhar GD (2005) Carbon isotope effect predictions for enzymes involved in the primary carbon metabolism of plant leaves. Funct Plant Biol 32:277–291

Tcherkez G, Hodges M (2008) How stable isotopes may help to elucidate primary nitrogen metabolism and its interaction with (photo)respiration in C_3 leaves. J Exp Bot 59:1685–1693

Tcherkez G, Nogues S, Bleton J, Cornic G, Badeck F, Ghashghaie J (2003) Metabolic origin of carbon isotope composition of leaf dark-respired CO_2 in French Bean. Plant Physiol 131:237–244

Tcherkez G, Mahe A, Gauthier P, Mauve C, Gout E, Bligny R, Cornic G, Hodges M (2009) In folio respiratory fluxomics revealed by 13C isotopic labeling and H/D isotope effects highlight the noncyclic nature of the tricarboxylic acid "cycle" in illuminated leaves. Plant Physiol 151:620–630

Thorpe MR, Minchin PEH (1991) Continuous monitoring of fluxes of photoassimilate in leaves and whole plants. J Exp Bot 42:461–468

Trumbore SE (2006) Carbon respired by terrestrial ecosystems – recent progress and challenges. Glob Change Biol 12:141–153

Trumbore SE (2009) Radiocarbon and soil carbon dynamics. Annu Rev Earth Planet Sci 37:47–66

Vargas R, Detto M, Baldocchi DD, Allen MF (2010) Multiscale analysis of temporal variability of soil CO_2 production as influenced by weather and vegetation. Glob Change Biol 16:1589–1605

Wild M (2010) The carbon and nitrogen supply systems of leaf growth in perennial ryegrass – characterization by dynamic ^{15}N and ^{13}C labelling and compartmental analysis of tracer influx into the leaf growth zone. Doctoral thesis, Technische Universität München

Yakir D, Wang XF (1996) Fluxes of CO_2 and water between terrestrial vegetation and the atmosphere estimated from isotope measurements. Nature 380:515–517