Tracing Changes in Ecosystem Function under Elevated Carbon Dioxide Conditions

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Responses of ecosystems to elevated levels of atmospheric carbon dioxide (CO₂) remain a critical uncertainty in global change research. Two key unknown factors are the fate of carbon newly incorporated by photosynthesis into various pools within the ecosystem and the extent to which elevated CO₂ is transferred to and sequestered in pools with long turnover times. The CO₂ used for enrichment in many experiments incorporates a dual isotopic tracer, in the sense that ratios of both the stable carbon-13 (¹³C) and the radioactive carbon-14 (¹⁴C) isotopes with respect to carbon-12 are different from the corresponding ratios in atmospheric CO₂. Here we review techniques for using ¹³C and ¹⁴C abundances to follow the fate of newly fixed carbon and to further our understanding of the turnover times of ecosystem carbon pools. We also discuss the application of nitrogen, oxygen, and hydrogen isotope analyses for tracing changes in the linkages between carbon, nitrogen, and water cycles under conditions of elevated CO₂.

Keywords: elevated carbon dioxide, stable isotopes, radiocarbon, global change, carbon cycle

The responses of terrestrial ecosystems to rising concentrations of atmospheric carbon dioxide (CO₂), and the resulting global changes, are still not fully understood. Humans emitted 6 gigatons of carbon per year into the atmosphere from fossil fuel burning and cement production during the 1990s, yet only about half of this carbon accumulated in the atmosphere. Of the remainder, about half was absorbed by the oceans and half by terrestrial ecosystems (IPCC 2001). The mechanisms that govern the magnitude of net carbon uptake by the terrestrial biosphere are unknown, but enhanced growth under elevated atmospheric CO₂ is commonly proposed (Schimel et al. 2000). Although individual plants often respond to elevated CO₂ by fixing more carbon in photosynthesis and thereby increasing production of biomass, this does not necessarily result in long-term removal of CO₂ from the atmosphere. Long-term removal requires the transfer of fixed carbon into long-lived ecosystem pools such as woody biomass and recalcitrant soil carbon (Körner 2000).

Researchers have exposed a variety of plant communities to elevated CO₂ levels to improve our understanding of whole-ecosystem responses. Such studies have used open-top chambers, closed chambers, natural CO₂ springs, and free-air CO₂ enrichment (FACE). These experiments are critical to our ability to determine the fate of carbon in the terrestrial biosphere. In addition to increasing the rate of fixation of carbon in photosynthesis, elevated atmospheric CO₂ levels may indirectly cause other changes in ecosystem function. Plants may, for example, reduce their uptake of water as a result of closing their stomata in response to elevated levels of atmospheric CO₂ (Morison 1998). The exchange of carbon and water with the atmosphere is also closely related to the cycling of nitrogen, which generally limits growth in temperate systems (Zak et al. 2000). Changes in the ratio of carbon to nitrogen in plant material may influence the quality of food available for herbivores. Thus, elevated atmospheric CO₂ levels can affect populations at different trophic levels (Lindroth 1996a, Owensby et al. 1996).

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The need to develop methods to quantify ecosystem responses to elevated CO₂ in relatively small-scale and short-term experiments presents both challenges and opportunities for ecologists. Measurements of the abundance of various carbon, hydrogen, oxygen, and nitrogen isotopes in samples have long been used in various subdisciplines of ecology as unique tools that integrate physical and physiological processes over space and time (table 1, box 1). New developments in mass spectrometry allow stable isotope analyses to be conducted using very small samples of biomass, soil organic matter, or water. These advances have improved our ability to make inferences about plant physiology, biogeochemistry, and ecosystem function. This is particularly valuable in studies of elevated CO₂, in which destructive measurements must be limited to maintain the integrity of experimental plots for long-term study. Many studies of elevated CO₂ effects are intrinsically isotopic tracer experiments because the ratios of carbon-13 (¹³C) to carbon-12 (¹²C) and of carbon-14 (¹⁴C) to ¹²C in the added CO₂ are distinct from the ratios in atmospheric CO₂. Plants grown in conditions of elevated CO₂ that contain such isotopic tracers will incorporate the tracer in carbon-containing compounds. The two labels can then be used to trace the fate of carbon newly incorporated into the ecosystem under high CO₂ conditions and to calculate the residence time of carbon in various pools.

Here we discuss current and potential research that combines isotope ecology and experiments with elevated CO₂ to improve our understanding of interactions between ecosystem function and global change.

### Table 1. Isotopes of common elements and their natural abundance.

<table>
<thead>
<tr>
<th>Element</th>
<th>Isotope</th>
<th>Abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen</td>
<td>²H</td>
<td>99.985</td>
</tr>
<tr>
<td></td>
<td>³H</td>
<td>0.015</td>
</tr>
<tr>
<td>Carbon</td>
<td>¹²C</td>
<td>98.89</td>
</tr>
<tr>
<td></td>
<td>¹³C</td>
<td>1.11</td>
</tr>
<tr>
<td></td>
<td>¹⁴C</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>¹⁴N</td>
<td>99.63</td>
</tr>
<tr>
<td></td>
<td>¹⁵N</td>
<td>0.37</td>
</tr>
<tr>
<td>Oxygen</td>
<td>¹⁶O</td>
<td>99.76</td>
</tr>
<tr>
<td></td>
<td>¹⁷O</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>¹⁸O</td>
<td>0.20</td>
</tr>
</tbody>
</table>

*Note: All the isotopes except ¹⁴C are stable.*

#### The carbon-13 label

The mean δ¹³C (see box 1) of atmospheric CO₂ is currently about −8 parts per mil (‰) (Keeling and Whorf 2002). In contrast, commercial CO₂ originating from geologically ancient carbon (e.g., carbon from petroleum byproducts or wells) is often relatively depleted in ¹³C, with δ¹³C values typically ranging from −27‰ to −45‰. In most experiments with elevated CO₂, the increase in CO₂ concentration is accomplished by mixing commercial CO₂ with ambient air. As a result, short-term fluctuations in canopy CO₂ concentration above and below the targeted concentration may occur, and the concentration may vary from one place to another. Assessing actual exposure of plants to CO₂, therefore, becomes a significant challenge. A further complication is that δ¹³C of the commercial CO₂ may vary if the source changes over time. Periodic sampling of the added CO₂ can track its isotopic composition, and subsequent modeling of CO₂ circulation in CO₂ plots can provide estimates of the average exposure of experimental plants to CO₂ and ¹³CO₂; however, the best way of measuring plants' CO₂ exposure over time may be to measure of isotopic ratios of carbon in C₄ plants (box 2; Pepin and Körner 2002). Such plants utilize a photosynthetic pathway in which the initial fixation of carbon occurs through the action of the enzyme phosphoenolpyruvate (PEP) carboxylase. This enzyme has a much lower isotopic fractionation factor than rubisco (ribulose bisphosphate carboxylase/oxygenase), the initial carbon-fixing enzyme for plants utilizing the more common C₃ photosynthetic pathway (O’Leary 1981, Farquhar et al. 1989); that is, PEP carboxylase discriminates less against ¹³C when it fixes carbon. The isotopic composition of C₄ plants therefore changes less in response to factors that influence stomatal opening (such as light, temperature, and humidity) than that of C₃ plants, and its variation tracks the isotopic content of atmospheric CO₂.

This property of C₄ photosynthesis is being used in a Swiss experiment in which a mature temperate forest is fumigated with CO₂ from tubing distributed throughout the canopy. C₄ plants placed in pots throughout the canopy are periodically sampled to calculate the effective atmospheric CO₂ concentration from δ¹³C of C₄ plant material (box 2; Pepin and Körner 2002). This method agrees well with estimates of average CO₂ level derived from measuring the CO₂ concentration in sampled air (figure 1) and greatly reduces the need for air turbulence modeling or rapid gas sampling devices while still providing spatial information about the CO₂ gradients that may occur in these experiments. Once the ¹³CO₂ exposure of experimental plants has been characterized, the ¹³C label can then be used to follow the fate of experimentally added CO₂ in the ecosystem.

#### The carbon-14 label

If commercial CO₂ is derived from fossil sources, it contains no measurable ¹⁴C, because it has effectively all decayed to nitrogen-14 (¹⁴N) during its long isolation from the natural source of ¹⁴C, the action of cosmic rays in the atmosphere and on Earth’s surface. (The half-life of ¹⁴C is 5730 years.) The absence of ¹⁴C in fossil CO₂ provides a useful isotopic tracer that may show less temporal variation than the ¹³C isotope ratio. When the ¹⁴C-free commercial CO₂ is added to ambient air, ¹⁴C concentrations in the air are diluted. This signal is present in the plots with elevated CO₂ in many completed and ongoing FACE experiments, but to date only one has attempted to take advantage of it. At the FACE experiment in Maricopa, Arizona, the ¹⁴C-depleted CO₂ added to experimental plots resulted in cotton plants that contained only 70% of the ¹⁴C activity of
plants in ambient plots—a change that is easily detectable with current methods. Organic carbon pools in soil from plots exposed to elevated CO2 were also depleted in 14C compared with soil from plots exposed to ambient CO2 (Leavitt et al. 1994). As with $\delta^{13}C$, any heterogeneity in plants and soils in the enriched plots induced by inhomogeneous broadcast of the commercial CO2 will lead to unevenness in the distribution of the tracer. Furthermore, because 14C is present in such small concentrations relative to 13C, and because very small samples are used in 14C analysis, effects of heterogeneity and contamination could be magnified in 14C relative to those for 13C.

The depleted 14C signal present in studies of elevated CO2 that used “dead” CO2 from petrochemical sources should not be confused with the 14C signal that was artificially generated during thermonuclear bomb testing and contributes to the current “natural” atmospheric background. This “bomb spike” created a background 14C time marker centered in 1964, after which the atmospheric 14C activity declined as the pulse was incorporated into the ocean, groundwater, plants, and soils. This time marker has been very helpful in understanding carbon cycling in soils, among other things (e.g., Dorr and Munnich 1986, Jenkinson et al. 1992, Trumbore 1993), quite apart from the value of 14C in experiments that employ fumigation with tracer CO2. If 14C depletion in plots with elevated CO2 is used as a tracer, it is with reference to current levels of atmospheric 14C in ambient plots, which, because of atmospheric tests of thermonuclear bombs, are still above 1950 levels.

**Box 1. Isotopes in ecology**

Isotopes are atoms of an element that differ in the number of neutrons. Heavier isotopes, having more neutrons, form stronger chemical bonds, have lower reaction rates in enzymatic reactions, diffuse more slowly, and are more often found in lower-energy phase states in equilibrium reactions, such as the phase between vapor and liquid water. These properties can be exploited to investigate physical and biochemical processes and are the foundation for stable isotope approaches in ecological studies. In addition, radioactive isotopes such carbon-14 ($^{14}C$) provide another tracer, particularly useful for dating biological material. Commonly used isotopes in ecology are given in table 1.

Isotopic composition is often expressed as a ratio between the heavier and the lighter isotope. Expression of the absolute ratio is impractical because of the large differences in natural abundance between isotopes of the same element (table 1), so it is more convenient to express an isotope ratio relative to a standard:

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

where $R$ is the molar ratio of the heavy to the light isotope and ‰ is parts per mil.

Isotope ratios are expressed relative to common international standards, such as the PDB (Pee Dee Belemnite) formation, a standard for the ratio of carbon-13 ($^{13}C$) to carbon-12 with an $R$ value of 0.0112372.

Isotopic composition can also be expressed as a discrimination ($\Delta$) against heavy isotopes in the reaction between source and product:

$$\Delta = \frac{R_{\text{source}}}{R_{\text{product}}} - 1.$$

To calculate $\Delta$ from $\delta$:

$$\Delta = \left( \frac{\delta_{\text{source}} - \delta_{\text{product}}}{1 + \delta_{\text{product}}/1000} \right).$$

In contrast, $^{14}C$ is expressed as an activity (concentration). Using conventional radiocarbon (gas proportional counting or liquid scintillation) or accelerator mass spectrometer $^{14}C$ dating methods, the $^{14}C$ activity is measured and expressed as percent modern carbon or as fraction modern carbon, in which, by convention, modern carbon activity is defined as the background activity in 1950 (Stuiver and Polach 1977). All activity measurements are corrected for any fractionation taking place in fixation by plants, soil processes, or laboratory procedures by normalizing to a standard $\delta^{13}C$ value of –25‰.

**Figure 1. Determination of experimental carbon dioxide (CO$_2$) concentration at the Swiss Canopy Crane site using two methods: air sampling and the carbon isotope composition of C$_4$ plants placed in the canopy (± 1 standard deviation). The square on the right refers to the average daytime CO$_2$ concentration across all enrichment days. Source: Pepin and Körner (2002).**
Discrimination of carbon-13 (13C) during photosynthesis in C₄ plants is caused by fractionation during diffusion through stomatal pores and fractionation by carbon-fixing enzymes, primarily rubisco (ribulose bisphosphate carboxylase/oxygenase). These effects were quantified by Farquhar and colleagues (1982):

\[
\Delta^{13}C_p = a + (b - a) \times c/c'_a
\]

where \(c/c'_a\) is the ratio of intercellular to ambient carbon dioxide (CO₂) partial pressure, \(a\) is the fractionation associated with diffusion (4.4 parts per mil [‰]), and \(b\) is the net enzymatic fractionation associated with carboxylation (27‰). Therefore, discrimination is a function of \(c/c'_a\), which is sensitive to a variety of factors that influence the balance of stomatal conductance and assimilation rate. Equation 1 (above) can also be combined with equation 3 from box 1 to predict the isotopic composition of biomass if the isotopic composition of the air is known.

For C₃ plants, we must separate fractionation factors for rubisco and phosphoenolpyruvate (PEP) carboxylase. The latter fixes carbon in the mesophyll for transport into bundle sheath cells. In these cells, rubisco is physically isolated from the stomatal cavity. There is no fractionation associated with a product if all of the substrate is utilized. Therefore, we need only consider the fractionation factor for rubisco to the extent that CO₂ or HCO₃⁻ (hydrocarbonate) leaks out of bundle sheath cells back into the stomatal cavity. These effects were quantified by Farquhar (1983):

\[
\Delta^{13}C_p = a + (b + b_4 \cdot \phi - a) \times c/c'_a
\]

where \(b_4\) is the fractionation associated with PEP carboxylase (–5.7‰), \(b_3\) is the fractionation associated with rubisco (30‰), and \(\phi\) is the leakiness of the bundle sheath. For many species, the term \((b_4 + b_3 \cdot \phi - a)\) is close to zero, such that \(\Delta^{13}C\) may show little environmental variation in C₃ plants (Farquhar et al. 1989). In this case, variability in \(\delta^{13}C_p\), which is related to \(\Delta^{13}C_p\) (box 1, eq. 3), can be interpreted as a function of \(\delta_{source}\) (see box 1). In using this approach, researchers should take care that \(\phi\) has been characterized in the species of interest.

Experiments with elevated CO₂. Carbon fixed in photosynthesis can be transferred into numerous ecosystem pools (figure 2). Will the increase in carbon fixed under elevated atmospheric CO₂ conditions be transferred into long-lived pools, or will the ecosystem carbon cycle merely be accelerated, resulting in no long-term net increase in stored carbon? Tracing the fate of isotopically distinct carbon fixed under elevated atmospheric CO₂ offers a unique means of resolving some of these uncertainties at the ecosystem scale.

Carbon inputs from isotopically labeled plants to the soils can be revealed by shifts in the isotopic composition of soil organic carbon. The larger the difference between the carbon isotopic composition of the plants and soils, the greater the potential for success in quantifying inputs and losses. This tracer signal has now been used in many FACE studies (Allison et al. 1983, Leavitt et al. 1994, 1996, Nitschelm et al. 1997, van Kessel et al. 2000, Leavitt et al. 2001, Schlesinger and Lichter 2001) and chamber studies (Hungate et al. 1997, Torbert et al. 1997) to determine the amount of new carbon that has been incorporated into soils during the course of an experiment. Such isotopic tracers, whether originating from ancient-source CO₂ introduced into experimental plots in FACE experiments or from contrived shifts between C₃ and C₄ vegetation that cause plants and soils to have distinct isotope ratios (the traditional approach), can then be further used to identify the specific organic carbon pools that new carbon has entered (Jastrow et al. 1996). One commonly cited limitation of this method in studies of elevated CO₂ is that there is typically no similarly strong isotope labeling in the control plots, so that comparing the difference in results between the elevated and control CO₂ treatments is not straightforward. This has been overcome by using:

- small subplots of soils within the control plots whose carbon isotope composition reflects previous growth of C₃ plants and is therefore distinct from that resulting from currently growing C₄ plants (Allison et al. 1983, Ineson et al. 1996, Cheng and Johnson 1998, Leavitt et al. 2001)
- small subplots exposed to pulsed pure 13CO₂ within control plots (Hungate et al. 1997, Leavitt et al. 2001)
- carbon dioxide labeled with 13C or 14C and used to fumigate entire control plots in chamber experiments (Lin et al. 1999, 2001)
- the small but quantifiable natural difference between 13C in control plants and in the local soil organic carbon (Nitschelm et al. 1997)
require the application of different experimental methods in the elevated CO₂ treatments and the control, so results should be interpreted with care.

Many experiments with elevated CO₂ have shown increased photosynthetic gain and larger biomass under elevated CO₂ conditions than under ambient conditions (Acklerly and Bazzaz 1995, Drake et al. 1997, DeLucia et al. 1999). However, another common response is an increase in respiratory losses of CO₂ from the soil (Zak et al. 2000). A critical element in understanding the significance of increased soil respiration is the partitioning between the autotrophic (plant-derived) and heterotrophic (soil organism–derived) origin of the respired carbon. When plants are larger, a proportional increase in autotrophic respiration is expected as a result of increased root biomass. Increased heterotrophic respiration may, however, be an indicator of an accelerated carbon cycle, in which plant carbon inputs are quickly decomposed after deposition as litter or senesced roots. Stable isotopes are increasingly being used to distinguish the origins of soil fluxes (Rochette and Flanagan 1997, Cheng and Johnson 1998, Andrews et al. 1999, Lin et al. 1999, 2001, Pendall et al. 2001) and provide an alternative to traditional harvest or disturbance methods (Edwards and Norby 1999, Högberg et al. 2001). It should be noted that exact partitioning of respiration between heterotrophic and autotrophic components is not possible with a single isotopic label, as the microbial community immediately surrounding the roots (the rhizosphere) may rapidly acquire the isotopic signature of labile root exudates. However, the substrate-induced respiration method, in which sugar derived from C₄ plants is applied to soil in an ecosystem containing C₃ plants (Högb begins and Ekblad 1996, Ekblad and Högb erg 2001), provides good estimates of the relative contributions of heterotrophic and autotrophic respiration, as autotrophic respiration will have a C₃ carbon isotope ratio distinct from microbial decomposition of the C₄ sugar. Furthermore, the use of C₄ plants and elevated CO₂ isotope tracers allows partitioning of the soil CO₂ flux into “old,” or pre-label, and “new,” or current growing season, components (e.g., Pendall et al. 2001).

The elevated ¹³CO₂ label has been used at the Duke FACE experiment in Durham, North Carolina. In this pine forest, soil respiration has increased in response to elevated CO₂ (Andrews and Schlesinger 2001). In 1998, soil respiration increased by about 25%, or 220 grams (g) of carbon per square meter (m²) per year, in the elevated CO₂ treatments. The heterotrophic component of this flux constituted an increase of 80 g carbon per m² per year, which corresponds well with the observation of Högberg and Högb erg (2002). According to the isotopic results, about 70% of the total increase in soil respiration in the plots with elevated CO₂ originated from carbon that was fixed since the beginning of the experiment in September 1996. Although the absence of an
isotope tracer prevents the use of a comparable partitioning approach in the control plots, these results are a clear indication that much of the extra carbon fixed under elevated CO₂ conditions may be transferred to short-lived carbon pools that are quickly returned to the atmosphere.

With the use of tracers in control plots, experiments using closed-system mesocosms have found that elevated atmospheric CO₂ stimulates decomposition of recently fixed carbon but suppresses decomposition of older soil organic matter (Lin et al. 2001), particularly in the presence of high nutrient availability (Calder et al. 1992, Cheng and Johnson 1998, Cardon et al. 2001). In the shortgrass steppe open-top chamber (OTC) experiment in Colorado, a change in the proportion of C₃ and C₄ plants, which occurred before the experiment began, caused currently growing plants and soil organic matter from previous vegetation to attain distinct isotope ratios. This has allowed researchers to apply stable isotopes to distinguish between root and rhizosphere respiration and heterotrophic decomposition in three treatments: chambers with elevated CO₂, chambers with ambient levels of CO₂, and nonchambered control plots. In this experiment, elevated CO₂ resulted in doubled decomposition rates but no change in root and rhizosphere respiration relative to ambient conditions (Pendall et al. 2003). In an OTC experiment on a California annual grassland, Hungate and colleagues (1997) found increases in both rhizosphere and heterotrophic respiration under conditions of elevated CO₂, with the enhancement of heterotrophic respiration originating primarily from the decomposition of senesced roots.

These experiments illustrate both the importance of examining changes in the whole-ecosystem carbon cycle in response to elevated CO₂ and the utility of carbon isotopes to serve as valuable tracers. Although plant biomass often increases under elevated CO₂, isotopic methods are providing a unique insight into changes in decomposition rates and the contribution of autotrophic and heterotrophic respiration. In general, isotopic evidence points toward greater heterotrophic respiration rates under elevated CO₂. In many studies, this increase has been attributed to greater decomposition of recently fixed labile carbon and to stimulation of microbial activity such that older carbon pools are also utilized. These results have important implications for the long-term sequestration of carbon in the terrestrial biosphere.

Quantifying the turnover rate of carbon in ecosystems

Quantifying the mean residence time of carbon in various ecosystem pools is a principal goal in several areas of carbon cycle research, particularly with regard to belowground fractions of biomass and soil organic matter (Randerson et al. 1999). In this regard, isotope analyses in experiments on elevated CO₂ provide an opportunity to advance our understanding of ecosystem function. These experiments are analogs of labeling studies previously conducted in small greenhouses or closed chambers. It is now possible to understand the time scales of carbon transfer through belowground carbon pools in whole-ecosystem manipulations using labeled CO₂.

In the Duke FACE experiment, within one week of the initiation of fumigation with depleted 13CO₂, the soil CO₂ efflux in experimental plots became isotopically depleted in 13C relative to the plots with ambient levels of CO₂ (Andrews et al. 1999), providing unequivocal evidence of the rapid carbon fluxes between aboveground and belowground components. This 13C depletion reflects transport of recently fixed carbon below ground, which caused a corresponding change in the isotope ratio of autotrophic respiration. Within a year of the initiation of fumigation, root-free soil showed 13C depletion in the CO₂ efflux, which must have been derived from the heterotrophic component of respiration. Yet after 32 days of incubation with no further supplemental carbon, the 13C-depleted respiration signal returned to values similar to those of soil from ambient plots. This indicated that the newly fixed carbon had been transferred to labile soil organic matter pools that were rapidly exhausted in the absence of continuous inputs (figure 3). These and other results support recent studies of naturally occurring 13C that show correlations between δ13C of soil-respired and ecosystem-respired CO₂ and environmental factors such as humidity and vapor pressure deficit (Ekblad and Högberg 2001, Bowling et al. 2002), illustrating that a large portion of respired carbon was recently fixed. These isotopic studies allow further delineation of the carbon cycle and provide strong evidence for rapid carbon cycling at the level of short-term photo-

![Figure 3. The carbon isotope ratio (δ13C) of soil carbon dioxide (CO₂) flux during root-free incubation of soil from the Duke Forest FACE (free-air CO₂ enrichment) experiment. Source: Andrews and colleagues (1999).](http://bioscience.oxfordjournals.org/)

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Carbon and nitrogen interactions

Ecosystem carbon and nitrogen cycles are tightly integrated, and isotope-based approaches can help elucidate changes under elevated CO₂ conditions. Organic material in the form of roots, litter, and woody debris is decomposed by the soil microbial community and releases mineral nitrogen available for plant uptake in the form of ammonium and nitrate (nitrogen mineralization). In temperate ecosystems, the availability of nitrogen is often a limiting factor for plant growth. To sustain increased growth under elevated CO₂ in these ecosystems, plants must either increase their nitrogen use efficiency (carbon fixed per unit of plant nitrogen) or increase their uptake of nitrogen. Increased nitrogen uptake may result from greater uptake of existing mineral nitrogen, from increased rates of mineralization, or from increased fixation by symbiotic or free-living organisms that can utilize nitrogen from the atmosphere and make it available to plants.

The number of possible alterations to the nitrogen cycle that could occur under elevated CO₂ introduces uncertainty into interpretations of experimental results. It is essential to determine whether short-term observations of increased plant growth under elevated CO₂ can be sustained in the future. This question hinges in part on an understanding of carbon and nitrogen interactions. To examine these interactions, ecologists have studied variations in naturally occurring levels of nitrogen-15 (¹⁵N) and conducted experiments using artificially applied ¹⁵N. These studies present considerable opportunities for understanding carbon–nitrogen interactions in experiments with elevated CO₂.

Table 2. Mean residence times of fine roots in the Duke Forest FACE (free-air carbon dioxide enrichment) site, estimated using carbon-13 and carbon-14 isotope tracers.

<table>
<thead>
<tr>
<th>Fine root class</th>
<th>Ambient carbon dioxide</th>
<th>Elevated carbon dioxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1 mm</td>
<td>4 to 5 years</td>
<td>4.2 years</td>
</tr>
<tr>
<td>1 to 2 mm</td>
<td>6 to 7 years</td>
<td>5.9 years</td>
</tr>
<tr>
<td>&gt;2 mm</td>
<td>8 to 9 years</td>
<td>6.0 years</td>
</tr>
</tbody>
</table>

Note: Incorporation of the isotope tracer into a given root size pool is measured as the quantity of roots that have the initial isotope ratios as a function of time fitted to an exponential function (F(t) = e⁻kt, mean residence time = 1/k years).
Lessons from natural abundance of nitrogen-15. The nitrogen isotope ratio ($\delta^{15}N$) is a useful indicator of change in ecosystem nitrogen cycles. The $\delta^{15}N$ of plant-available nitrogen is a function of several variables, including nitrogen inputs into the ecosystem, $\delta^{15}N$ of substrates used by soil microbial populations, rates of microbial transformations, gaseous losses from the soil, and changes in active plant rooting depths. Changes in any of these factors as a result of growth under elevated CO$_2$ should alter the $\delta^{15}N$ of nitrogen available to plants and subsequently the $\delta^{15}N$ of the plants themselves. Therefore, a significant shift in plant $\delta^{15}N$ can be a conclusive indicator of changes in the ecosystem nitrogen cycle long before small shifts in the overall nitrogen content of different ecosystem components can be detected.

Recently, a large shift in plant $\delta^{15}N$ under simulated conditions of elevated CO$_2$ was observed at the Nevada Desert FACE facility. Billings and colleagues (2002) documented a 3‰ increase in $\delta^{15}N$ of the dominant shrub (Larrea tridentata) following exposure to elevated CO$_2$ and seasonal shifts of 2‰ for shrubs grown under both ambient and elevated CO$_2$ conditions. In September 1999, four shrub species showed enrichment in $\delta^{15}N$ under conditions of elevated CO$_2$ relative to ambient conditions (figure 4). No significant differences were observed in root growth and water sources between the two treatments. Moreover, plant $\delta^{15}N$ was actually greater than soil $\delta^{15}N$, suggesting enrichment of $^{15}N$ in the pool of nitrogen available to plants as a result of mineralization. This could be caused by an increase in gaseous nitrogen loss or by immobilization in microbial biomass, because both processes are accompanied by significant fractionation, that is, preferential loss of $^{14}N$ and enrichment of the remaining pool in $^{15}N$ (Högberg 1997). The shift in plant $\delta^{15}N$ and other supporting evidence in the Nevada study strongly suggest an increase in microbial activity under conditions of elevated CO$_2$ associated with the greater inputs of organic substrate available for decomposition.

The numerous transformations that occur in the nitrogen cycle prohibit $^{15}N$ at natural abundance levels from being used as a tracer of nitrogen movement in ecosystems (Evans 2001, Robinson 2001). One exception is that $^{15}N$ discrimination is not observed during plant uptake of inorganic nitrogen at typical soil concentrations; therefore, whole-plant $\delta^{15}N$ will accurately trace that of the source if plants absorb ammonium or nitrate (Mariotti et al. 1982, Yoneyma and Kaneko 1989, Evans et al. 1996). A complication is that $^{14}N$ fractionation may occur during mycorrhizal uptake and transfer of nitrogen to the host, but $^{15}N$ discrimination will not be observed if most of the nitrogen absorbed by the fungus is transferred to the host (Hobbie et al. 1999).

Lessons from tracer nitrogen-15. An alternative approach to the use of natural abundance nitrogen isotopes is to study nitrogen cycling with the application of an artificial $^{15}N$-enriched tracer. An important consideration is that the application of an artificial tracer excludes the possibility of subsequent studies of natural abundance, even if the tracer is applied to a small subsection of the experimental plot. Artificial $^{15}N$ tracers are far more enriched in $^{15}N$ than naturally occurring organic material, with $^{15}N$ concentrations that are 1000 to 10,000 times higher. It is difficult to avoid contamination under these circumstances.

One of the strengths of the $^{15}N$ tracer technique is the possibility of constructing a complete $^{15}N$ budget by following the tracer into the various plant and soil pools. This provides an opportunity to address the question of whether elevated CO$_2$ leads to greater nitrogen use efficiency of plants, increased available nitrogen, or decreased nitrogen losses from the plant–soil system. Increases or decreases in nitrogen availability could occur depending on whether elevated CO$_2$ stimulates or suppresses soil organic matter decomposition. A number of studies have assessed how plant nitrogen use and total $^{15}N$ losses have been altered under elevated CO$_2$ conditions. Losses of applied $^{15}N$ were found to be slightly lower under elevated CO$_2$ in a microcosm study when the grass Danthonia richardsonii was grown under a high level of applied mineral nitrogen (Lutze and Gifford 1998). A FACE experiment that made use of $^{15}N$-labeled fertilizer indicated that elevated CO$_2$ concentration had no significant effect on total $^{15}N$ losses (soil and plant) in swards of Lolium perenne (perennial ryegrass) and Trifolium repens (white clover) after 4 years of CO$_2$ enrichment (Hartwig et al. 2002). These studies suggest that the total nitrogen in the
plant–soil system is not altered under elevated CO₂ in these ecosystems. Moreover, in the *Lolium perenne* system after 8 years of elevated and ambient CO₂ concentrations, ¹⁵N enrichments were similar in the various soil organic matter fractions, showing that the soil organic matter–nitrogen dynamics were unaffected by prolonged exposure to elevated CO₂ (van Groenigen et al. 2002).

Nitrogen fixation plays a key role in the overall nitrogen cycle, and the total input in terrestrial ecosystems is estimated at around 100 teragrams per year (Mosier 2001). Estimates for molecular nitrogen (N₂)–fixing activity in the field are based on the dilution of ¹⁵N in N₂-fixing legumes compared with non-N₂-fixing reference plants. Using the ¹⁵N dilution approach, the percentage of nitrogen derived from N₂ fixation by *T. repens* grown under elevated CO₂ (FACE) conditions increased by 8% over a 3-year period (Zanetti et al. 1996). Similar findings were reported for free-living, N₂-fixing bacteria in stands of a C₃ sedge and a C₄ grass grown under elevated CO₂ (Dakora and Drake 2000). It will be of interest to determine whether the increase in N₂ fixation under elevated CO₂ conditions will be sustained. Once the feedback mechanisms between the demand of nitrogen by the plant, available soil nitrogen, and N₂-fixing activity have reached a new equilibrium under elevated CO₂, the increase in N₂ fixation may subside.

### Integrating altered water balance

In water-limited ecosystems, water availability has been shown to be the critical variable controlling plant growth responses under elevated CO₂ (Mooney et al. 1999). Elevated CO₂ can improve plant water relations, increase soil water availability, and indirectly alter organic matter decomposition, nutrient cycling, and carbon storage. In addition, alterations to evapotranspiration and biosphere–atmosphere exchange of water vapor may have important implications for climate and the hydrologic cycle. Stable isotopes of carbon, oxygen, and hydrogen record the influence of environmental conditions and plant water sources on plant function. Isotopic water balance studies take advantage of the large environmental gradients in the isotopic composition of precipitation and soil water. Such gradients provide natural tracers that avoid the need for artificial labeling (figure 5). Unlike short-term measurements of leaf gas exchange and temperature, isotopic methods may be used to study long-term changes in plant energy and water balance.

### Lessons from carbon isotopes

The carbon isotope ratio of plant organic matter (δ¹³C_p) is an integrated measure of photosynthetic discrimination (Δ¹³C) during CO₂ fixation. The parameter Δ¹³C takes into account variations in δ¹³C of source air (δ¹³C_source; box 1) in the formation of plant material. It is particularly important to take into account δ¹³C_source in studies of elevated CO₂, in which δ¹³C_source is often significantly different from ambient levels. When effects of δ¹³C_source are removed, Δ¹³C_p in C₃ plants is a function of the ratio of the partial pressures of CO₂ inside and outside the leaf (c/c_i; box 2). This ratio reflects the balance between CO₂ diffusion into the leaf as regulated by stomatal conductance and CO₂ removal as regulated by the photosynthetic rate. Therefore, the c/c_i ratio can be considered an integrated set point of plant gas exchange (Ehleringer and Cerling 1995).

By applying estimates of δ¹³C_source and measurements of δ¹³C_p to estimating c/c_i integrated over time in studies of elevated CO₂, we can assess the effects of elevated CO₂ on this set point, that is, how stomatal regulation of photosynthetic gas exchange has responded to a higher-CO₂ environment.

Short-term, instantaneous leaf-level estimates of c/c_i can be obtained with traditional gas exchange measurements. While several studies have reported no short-term changes in instantaneous c/c_i under elevated CO₂ (see Morison 1985, Drake et al. 1997), longer-term measurements using δ¹³C_p to estimate assimilation-weighted c/c_i values have suggested otherwise. One of the authors (G. L.) estimated c/c_i values from plants grown under elevated CO₂ in a variety of ecosystem facilities, including chambers, micro- and mesocosms, FACE experiments, and CO₂ springs, and concluded that about two-thirds of studies examined showed significant increases in the c/c_i ratio under elevated CO₂, while the rest showed no change in c/c_i. This is a key observation, because long-term increases in c/c_i are associated with increased water-use efficiency, defined as the ratio of carbon fixed to water lost (Farquhar et al. 1989). These δ¹³C measurements will not only help resolve uncertainty in physiological changes in response to rising CO₂ concentrations over the long term but also improve parameterization of ecological models, for which c/c_i is often a critical variable (see Katul et al. 2000).

### Lessons from oxygen and hydrogen isotopes

The isotopic composition of plant stem water reflects the isotopic composition of the plant’s water source, because, with the exception of several halophytes (Lin and Sternberg 1993), there is no fractionation against the heavy hydrogen isotope deuterium and oxygen (δ¹⁸O) isotopes during plant water uptake (White et al. 1985, Dawson and Ehleringer 1991, Lin and Sternberg 1993). However, because of evaporative effects, leaf water becomes isotopically enriched in δ¹⁸O by 15‰ to 30‰ and in deuterium by 40‰ to 60‰ relative to stem water (box 3). In the leaf, oxygen in CO₂ readily exchanges with oxygen in liquid water, a rapid reaction in the presence of the plant enzyme carbonic anhydrase. Because plant leaves contain much more water than CO₂, the δ¹⁸O of CO₂ takes on the δ¹⁸O value of leaf water. Farquhar and colleagues (1993) and Farquhar and Lloyd (1993) have shown how measurements of the δ¹⁸O values of leaf water and CO₂ can be used to estimate gross photosynthetic rates. In addition, organic matter fixed within leaves (i.e., sugars and cellulose) records the enriched δ¹⁸O values of leaf water. Because δ¹⁸O and deuterium have similar applications and are subject to similar mechanisms of evaporative enrichment, we will restrict our discussion below to δ¹⁸O for convenience.

Models predicting the isotopic composition of leaf water and plant cellulose are shown in box 3. These
Although there is no fractionation in plant water uptake or transport (White et al. 1985), leaf water is isotopically enriched relative to the plant water source because of evaporation. To quantify this effect, plant leaves can generally be treated as evaporation pools of water. However, unlike large water reservoirs, leaves have a high ratio of evaporative flux to water volume and have nearly equal amounts of water entering from the xylem and leaving from evaporation. Under these steady-state conditions, we can predict the isotopic composition of leaf water at the site of evaporation with a simplified version of the model given by Craig and Gordon (1965):

\[ \delta_{Ls} = \delta_a + \epsilon_{eq} + \epsilon_k + (\delta_a - \delta_s - \epsilon_k) \times e_i/e_a, \]

where \( \delta \) stands for \( \delta^{18}O \); the subscript \( a \) is ambient water vapor; \( \epsilon_{eq} \) is the equilibrium fractionation, which is temperature dependent (Majoube 1971); \( \epsilon_k \) is the kinetic fractionation of water, which depends on the molecular diffusion of \( ^{18}O \) in air and the aerodynamic nature of the boundary conditions (Merlivat 1978); and \( e_i/e_a \) is the ratio of the partial pressure of water vapor outside and inside the leaf (Farquhar and Lloyd 1993, Yakir 1998). The \( ^{18}O \) signal from leaf water at the site of evaporation is transferred to organic matter (mostly cellulose) via biosynthesis from sucrose and starch with an averaged oxygen fractionation value of 27%o (DeNiro and Epstein 1979, Sternberg et al. 1989, Yakir 1992).

Although equation 1 (above) is useful for predicting the isotopic composition of water at the site of evaporation, it is often inadequate to predict the isotope ratio of bulk leaf water. Not all parts of the leaf are equally exposed to evaporation, and significant spatial heterogeneity within leaves has been found (Yakir et al. 1989, Luo and Sternberg 1992, Yakir et al. 1994, Helliker and Ehleringer 2000). Some of this heterogeneity may be caused by patchiness (Terashima et al. 1988), leaf compartmentalization (Yakir et al. 1993), and incomplete mixing away from the site of evaporation at the cell walls (Farquhar and Lloyd 1993). To incorporate the effect of the decay of the \( ^{18}O \) enrichment signal between the surface of evaporation and the isotopic signature of the source water entering the leaf, Farquhar and Lloyd (1993) proposed

\[ \delta_{LW} = \delta_a + (\delta_{Ls} - \delta_a) \times (1 - e^{-P}) / P, \]

as modified by Yakir (1998), where \( \delta_{LW} \) is the \( ^{18}O \) bulk leaf water, \( \delta_a \) in equation 1, and \( P \) is the Péclet number. \( P \) is related to the transpiration rate (moles per square meter per second) and the effective maximal mixing path length as well as physical constants. An alternative approach to estimating transpiration rate to determine \( \delta_{LW} \) has been proposed by Roden and Ehleringer (1999), who apply best-fit empirical coefficients to leaf water mixing.

Equations can be used to interpret the effects of elevated \( CO_2 \) on \( ^{18}O \) of bulk leaf water, which may occur as a result of several possible changes in plant function and canopy structure. Leaf water of plants grown under elevated \( CO_2 \) can be expected to become enriched in \( ^{18}O \) if transpiration is reduced (box 3, eq. 2), and even more so if this reduction causes an increase in leaf temperature and therefore an increase in the leaf saturation vapor pressure (box 3, eq. 1). Conversely, reduced enrichment is expected if transpiration increases under elevated \( CO_2 \) because of increased plant size and rooting volume (box 3, eq. 2).

Preliminary results from a Swiss forest experiment showed that leaves of deciduous trees exposed to elevated \( CO_2 \) were enriched in \( ^{18}O \) relative to those grown under ambient \( CO_2 \). However, the humidity, leaf temperature, and air temperature in the canopy under elevated \( CO_2 \) did not differ significantly from ambient conditions. Thus, it is likely that reduced transpiration under elevated \( CO_2 \) caused the enrichment in \( H_2^{18}O \) (see box 3, eq. 2). Cooper and Norby (1994) found species-specific effects on \( H_2^{18}O \) enrichment: Both Quercus alba and Liriodendron tulipifera showed reductions in transpiration under elevated \( CO_2 \), but only Li. tulipifera showed \( ^{18}O \)-enriched leaf water and leaf cellulose (figure 6). These results demonstrate that subtle changes in water and energy balance, which may be difficult to detect with traditional meteorological and physiological measurements, may occur in plants grown under elevated \( CO_2 \). Isotopic approaches allow detection of the integrated effects over time of changes in the isotopic composition of leaf water and organic components. In addition, these studies have consequences for the interpretation of changes in isotope ratios in long-term data sets, such as tree ring data, that encompass historical changes in atmospheric \( CO_2 \) concentrations.

A mechanistic assessment of the effects of elevated \( CO_2 \) on water fluxes is facilitated by the use of stable isotopes in soil water. Soil water near the surface undergoes evaporation and becomes \( ^{18}O \) enriched, whereas water from deeper layers is unchanged isotopically from precipitation inputs. Since plants do not fractionate soil water during uptake, and water vapor leaving the leaf is in isotopic equilibrium with stem water, water evaporating from soil and water transpired by plants often have isotopically distinct compositions. This difference affords a means of comparing the magnitudes of the soil and plant components of evapotranspiration.

This approach was applied at the Colorado OTC experiment, which had as its subject a perennial grassland. In this study, the oxygen isotope ratio of soil \( CO_2 \) was used as a proxy for \( ^{18}O \) of soil water. Measurements of \( ^{18}O \) in soil
CO₂, soil water, and precipitation, as well as the amount of precipitation, were used in a mass balance model to assess evaporation and transpiration (Ferretti et al. 2003). Soil water δ¹⁸O in chambers with ambient CO₂ was more enriched than in chambers with elevated CO₂. The mass balance showed that this enrichment was in part a consequence of year-to-year variability in the effects of elevated CO₂ on ecosystem water balance. During an unusually dry growing season, transpiration in chambers with elevated CO₂ was higher than in those with ambient CO₂, reflecting the larger plant biomass under elevated CO₂ conditions. In years of average precipitation, however, transpiration was similar under both treatments. Despite these differences, transpiration use efficiency, defined as the amount of biomass fixed per unit of transpiration, was greater in chambers with elevated CO₂ in dry and normal years. These results illustrate a unique application of stable isotope tracers to understanding changes in ecosystem water use under elevated CO₂. Such applications could be particularly valuable in light of the difficulties in distinguishing the components of evapotranspiration with nonisotopic methods.

Trophic-level interactions
Changes in plant tissue composition and chemistry induced by elevated CO₂ have important implications not only for carbon and nutrient cycles but also for interactions between plants and communities of organisms at higher trophic levels. Although there have been few studies of the effects of elevated CO₂ on fauna, effects on leaf carbon-to-nitrogen (C:N) ratios, and on the levels and amounts of the secondary metabolic compounds that deter herbivory, have been shown to change leaf consumption by herbivorous insects and consequently the insects’ growth rate (Lindroth 1996a, 1996b). There is considerably less information available on the effects of elevated CO₂ on the chemistry of fine roots and root exudates, but such changes have been hypothesized to have a strong influence on soil microbial community dynamics and subsequently on the processes of nitrogen mineralization and immobilization that control the availability of nitrogen to plants (Zak et al. 2000).

The isotopic composition of animals reflects that of their food source when tissue-dependent metabolic fractionation effects have been accounted for (DeNiro and Epstein 1978, Tiezen et al. 1983, Focken and Becker 1998). Therefore, stable carbon isotopes have been used extensively in studies of terrestrial, aquatic, and marine food webs (Rounick and Winterbourn 1986). Carbon dioxide fumigation with a highly ¹³C-depleted source creates a unique isotopic signature in plant material, which can be traced to higher trophic levels. This provides a unique opportunity to evaluate changes in diet in a high-CO₂ environment.

In a novel application of this idea, Cotrufo and Drake (2000) collected litter from several FACE experiments with contrasting δ¹³C values of approximately −27‰ in plots with ambient levels of CO₂ and −40‰ in plots with elevated levels of CO₂. Litter from plots with elevated CO₂, litter from plots with ambient CO₂, and a 50–50 mixture of the two were then inoculated with isopods and incubated. The CO₂ respired from each incubation was collected and analyzed for ¹³C. The results showed that CO₂ respired from the 50–50 mixture had a carbon isotope ratio of 34‰, an amount intermediate between the ratios in CO₂ evolved from litter from plots with ambient and with elevated CO₂. The results indicate that the detritivores showed no preference for litter from plots with ambient CO₂ over litter from plots with elevated CO₂. This finding contrasts with hypotheses based on the commonly observed increase in leaf C:N ratios under elevated CO₂. These hypotheses propose that decomposition should occur more slowly in litter with a higher C:N ratio, reducing the rate of litter decomposition under elevated CO₂ (Strain and Bazzaz 1983). However, the results were consistent with a recent meta-analysis that found no overall CO₂ effect on litter decomposition rates from the range of results reported to date (Norby et al. 2001), despite the differences in live leaf C:N ratios and the increased levels of secondary compounds, both of which affect herbivory, in plants grown under elevated CO₂. Similar approaches may be used to understand herbivore preferences for plant material grown under elevated CO₂ and ambient conditions.
In addition to the CO₂ fumigation tracer, other labeling techniques have been used to examine the flow of carbon into the soil microbial community under elevated CO₂. One such technique is pulse labeling with ¹⁴C. In this technique, plants are exposed to a brief pulse of CO₂ containing elevated ¹⁴C. In an experiment on the effects of elevated CO₂ on crops, pulse labeling showed that high CO₂ caused reduced allocation of carbon into the microbial carbon pool, probably the result of a change in the types and amounts of carbon compounds available to microbes under elevated CO₂ (Patterson et al. 1996). It is also likely that alterations in the amount and composition of carbon deposited to the soil can affect microbial community composition as well as biomass, but this has been difficult to assess. Radajewski and colleagues (2000) reported that taxonomic groups with distinct metabolic processes could be distinguished by supplying a ¹³C-enriched substrate to the soil. Microbes that utilize the substrate incorporate the isotope into their DNA, and density-gradient centrifugation can then be employed to separate DNA containing the added ¹³C isotope from DNA without the added isotope. Sequence analysis of the DNA thus separated can identify the phylogenetic group that metabolizes a particular substrate. This technique holds promise for evaluating shifts in microbial community composition in response to environmental perturbations such as elevated CO₂. Thus, a variety of isotopic tracers can be used to evaluate hypothesized shifts in plant–animal interactions and microbial food webs resulting from changes in plant chemistry.

Conclusions
Carbon, nitrogen, oxygen, and hydrogen isotope ratios at natural abundance levels provide valuable tracers for integrated analyses of terrestrial ecosystems in experiments with elevated levels of CO₂. The additional CO₂ supplied in these experiments provides a useful label that can be used to trace carbon flows and turnover rates in different pools, both under elevated CO₂ treatment conditions and after supplemental CO₂ is withdrawn. In experiments in which isotope ratio mass spectrometry is not available, dry biomass and soil samples can be archived for future isotopic analysis. Frozen leaf and soil samples or, if possible, water extracted and stored in sealed vials can provide valuable information about the effect of elevated atmospheric CO₂ on leaf water budgets and possibly on leaf energy balance. Linking these stable isotope analyses with traditional methods in elevated-CO₂ ecosystem studies provides a unique opportunity for tracing short-term and long-term changes in the carbon cycle and their impacts on key ecosystem biogeochemical cycles as well as trophic-level interactions.

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