

# Stable isotope approaches and opportunities for improving plant conservation

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Successful conservation of threatened species and ecosystems in a rapidly changing world requires scientifically sound decision-making tools that are readily accessible to conservation practitioners. Physiological applications that examine how plants and animals interact with their environment are now widely used when planning, implementing and monitoring conservation. Among these tools, stable-isotope physiology is a potentially powerful, yet under-utilized cornerstone of current and future conservation efforts of threatened and endangered plants. We review the underlying concepts and theory of stable-isotope physiology and describe how stable-isotope applications can support plant conservation. We focus on stable isotopes of carbon, hydrogen, oxygen and nitrogen to address plant ecophysiological responses to changing environmental conditions across temporal scales from hours to centuries. We review examples from a broad range of plant taxa, life forms and habitats and provide specific examples where stable-isotope analysis can directly improve conservation, in part by helping identify resilient, locally adapted genotypes or populations. Our review aims to provide a guide for practitioners to easily access and evaluate the information that can be derived from stable-isotope signatures, their limitations and how stable isotopes can improve conservation efforts.

**Key words:** plant water-use efficiency, plant water sources, local adaptation, global environmental change, climate reconstruction

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## Introduction

Conservation physiology is defined as ‘the study of physiological responses of organisms to human alteration of the environment that might cause or contribute to population declines’ (Cooke *et al.*, 2013). Conservation physiology goes beyond the description of empirical patterns and instead

establishes underlying mechanisms for population declines. The field of conservation physiology is well established as an important tool to shape conservation priorities in a wide range of animal taxa in aquatic, marine and terrestrial ecosystems (Cooke *et al.*, 2013). However, linkages between physiology and plant conservation are not as well established as they are in animal conservation, although emerging opportunities

to guide plant conservation through physiology are becoming more widely recognized (Richards *et al.*, 2003; van Kleunen, 2014; Madliger *et al.*, 2018). With almost all plant taxa, fitness (i.e. growth, survival and fecundity) is largely governed by photosynthetic processes (e.g. nutrient transport, stomatal conductance, sunlight capture) and non-photosynthetic processes (e.g. respiration, water transport, phloem transport, starch storage, synthesis of secondary metabolites). A wide range of physiological tools have been used to quantify how photosynthetic and non-photosynthetic metabolic processes respond to environmental alteration. For example, sensors are now widely used to quantify rates of water transport from soils to leaves, photosynthesis and respiration (Madliger *et al.*, 2018). Here we examine how the use of stable isotopes complements the use of sensors and other tools to expand the physiological toolbox for plant conservation.

Stable-isotope ratios or ‘signatures’ in plant tissues, metabolites and xylem water are integrators of physical, chemical and biological processes (Fry, 2006). They are also tracers that follow the fate and transformations of resources as they are acquired by plants and transported through various trophic levels within ecosystems (Dawson *et al.*, 2002). Plant physiologists have used stable-isotope ratios to determine photosynthetic and respiratory pathways, monitor plant stress, identify the sources of water and nitrogen used by plants, estimate water and nitrogen use efficiencies and reconstruct past climates from plant tissues (Rundel *et al.*, 1989; Ehleringer and Dawson, 1992; Erskine *et al.*, 1998; McCarroll and Loader, 2004; Ribas-Carbo *et al.*, 2005; Watling *et al.*, 2006; Wasley *et al.*, 2012; Cernusak, 2020). Stable isotopes can shed light on plant resource acquisition under multiple stressors and across a range of spatial and temporal scales. As a consequence, stable-isotope techniques are considered among the most important empirical tools in plant physiological ecology over the last forty years (Dawson *et al.*, 2002).

In this overview, we focus on stable isotopes of hydrogen, oxygen, carbon and nitrogen as short-term and long-term indicators of plant physiological performance, and how these indicators add to the toolbox used for the conservation of plants and their ecosystems. A full description of the basic theory, methods and terminology is beyond our scope (see Dawson *et al.*, 2002; Fry, 2006; Gannes *et al.*, 1998; McCarroll and Loader, 2004; Peterson and Fry, 1987, for in-depth reviews of stable-isotope theory and utility), rather we discuss applications in which stable-isotope physiology can support conservation. We provide a general framework illustrating how stable-isotope signatures in plant tissues vary across short-, mid- and long-term temporal scales and how knowledge of the signatures can support conservation efforts (Table 1). Plant tissues and biochemicals analysed for stable-isotopes include photosynthetic tissues such as whole leaf in vascular plants and whole plant in non-vascular taxa; structural tissues such as tree-ring cellulose and cactus spines; water extracted from plant xylem tissues; plant compounds


such as leaf sugars, lipids and waxes; and products of photosynthesis and respiration such as respired CO<sub>2</sub>. This framework highlights how stable-isotope signatures can be useful ecological indicators of stressors that may ultimately produce ecological consequences (Table 1). Understanding these ecological indicators can aid in developing effective adaptive management strategies. As more stable-isotope datasets become available, and advances in isotope mass spectrometry continue to emerge, stable-isotope analyses and applications will continually become more accessible to conservation biologists and managers.

To highlight this point, we have identified 18 questions from the list of 100 research questions in conservation physiology recently identified by Cooke *et al.* (2021) in which stable-isotope applications can support conservation practitioners (Table 2). Specifically, we have identified at least 17 questions that can be addressed through  $\delta^{13}\text{C}$  analysis of plant tissues, at least 6 questions that can be addressed through analysis of  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  in plant/soil/atmospheric water, 5 questions that can be addressed through  $\delta^{15}\text{N}$  tissue analysis and 3 that can be addressed through analysis of  $\delta^{18}\text{O}$  in tissues such as tree-ring cellulose (Table 2). Indeed, the list in Table 2 should not be considered exhaustive as stable isotopic tools have potential to address further questions in conservation physiology (Cooke *et al.*, 2021), and we added two additional questions suitable for stable-isotope applications. Throughout this review, we highlight the questions of Cooke *et al.* (2021) and provide context for future research that couples conservation to stable-isotope physiology.

## Isotope theory in biological applications

Plant physiologists developed the application of stable-isotope techniques to study the interactions between plants and their environment. Isotopes of a given element differ in the number of neutrons they contain and are classified as being either ‘heavy’ or ‘light’ depending on whether they are neutron rich or poor, respectively. Ecologists and biologists take advantage of the fact that small differences between heavy and light isotopes of a given element make their rate of reaction, or the partitioning of products, vary in physical or chemical processes depending on the energetics of the process/reaction. Therefore, the ratios between heavy and light isotopes can yield information on a wide range of environmental and physiological processes. The stable-isotope concentrations of a sample are expressed as the molar ratio of the heavy to light isotopes. Since this ratio is small, stable-isotope abundances of a sample ( $R_{\text{sample}}$ ) are expressed relative to an internationally recognized standard ( $R_{\text{standard}}$ ) and multiplied by 1000 to yield units in per mil (‰). Isotope ratios are described in delta ( $\delta$ ) notation, where  $\delta X = ((R_{\text{sample}}/R_{\text{standard}}) - 1) \times 1000$ , such that X denotes the heavy isotope of a pair. A positive  $\delta X$  value means that the sample contains more of the heavy isotope than the standard;

**Table 1:** Examples of how stable-isotope ratios can be used in conservation physiology, across increasing temporal scales

| Isotopes   | Increasing temporal scales    |  |   |
|--|---|--|---|
| $\delta^2\text{H}$ & $\delta^{18}\text{O}$                             | Seasonal plant water source use<br>Measurement: isotopic signatures in xylem water and soil water   | Forensic evidence of illegal plant trade<br>Measurement: isotopic signatures in plant tissues and precipitation                                    | Plant provenances & isoscape mapping<br>Measurement: isotopic signatures in plant tissues and precipitation   |
| $\delta^{13}\text{C}$  | Daily response to drought and heatwaves<br>Measurement: $\delta^{13}\text{C}$ signatures of leaf photosynthates   | Seasonal legacy effects of drought and heatwaves<br>Measurement: $\delta^{13}\text{C}$ signatures of whole-leaf tissue or individual cactus spines | Inter-annual legacy effects of drought and heatwaves<br>Measurement: $\delta^{13}\text{C}$ of annual growth rings or down moss shoots and peat layers                   |
| $\delta^{13}\text{C}$ & $\delta^{18}\text{O}$                          | Seasonal stomatal sensitivity to drought and heat stress<br>Measurement: isotopic signatures of whole-leaf tissue or cactus spines  | Inter-annual stomatal sensitivity to drought and heat waves<br>Measurement: isotopic signatures of annual growth rings or cactus spines            | Long-term climate reconstructions<br>Measurement: isotopic signatures of annual growth rings, or cactus spines constructed over decades, or moss and peat               |
| $\delta^{15}\text{N}$<br>$\delta^{15}\text{N}$ & $\delta^{18}\text{O}$ | Plant N use in habitat with isotopically distinct N sources<br>Measurement: isotopic signatures of whole-leaf tissues or xylem water and N sources ( $\text{NO}_y$ , $\text{NH}_x$ in air, soil, water) | Nitrogen cycle<br>Measurement: isotopic signatures of plant tissues (leaves, wood) and N sources   | Historic trends of N sources and cycling, paleo-environment reconstruction<br>Measurement: isotopic signatures of plant tissues including recent, herbarium and fossils |

a negative  $\delta X$  value means that the sample contains less of the heavy isotope than the standard.

Here we focus on the stable-isotope ratios of (i) hydrogen  $^2\text{H}/^1\text{H}$  ( $^2\text{H}$  is also called deuterium and then ratio is presented as  $\delta\text{D}$ , but is the same as  $\delta^2\text{H}$ ); (ii)  $^{18}\text{O}/^{16}\text{O}$ ; (iii) carbon  $^{13}\text{C}/^{12}\text{C}$ ; and (iv) nitrogen  $^{15}\text{N}/^{14}\text{N}$ . The natural abundance levels of these isotopes, as well as studies using artificially enriched stable-isotope tracers, can provide information to guide conservation efforts (Peterson and Fry, 1987; Dawson *et al.*, 2002). Changes in the relative abundance of heavy versus light isotopes between a source substrate and the product(s) are called isotopic fractionation (see Table 3 for the definition of common terms). There are two primary types of fractionation: equilibrium and kinetic fractionation. Equilibrium isotope fractionation occurs during reactions that change phases (e.g. going from water vapour to liquid water) and are temperature dependent. These reactions can go both forward and backward depending on the energetics of the phase changes. In the example of water going from vapour to liquid, the heavier isotopes become enriched in the liquid water relative to the vapour. Kinetic fractionation is generally unidirectional, and the reaction rates are mass dependent. Many biological processes are unidirectional and, therefore, isotopic fractionations in biotic systems are usually caused by kinetic fractionation. Many biological kinetic fractionations are mediated by an enzyme that discriminates against an isotope in a mixture (for more details, see Dawson *et al.*, 2002; Kendall and Caldwell, 1998; Peterson and Fry, 1987). For example, in  $\text{C}_3$  plant photosynthesis the enzyme Rubisco discriminates against  $^{13}\text{C}$ , thus producing metabolites and

tissues that are depleted in  $\delta^{13}\text{C}$  relative to the atmosphere (current atmospheric  $\text{CO}_2$  approximately  $-8.5\text{‰}$ ). Carbon stable-isotope ratios ( $\delta^{13}\text{C}$ ) in plant tissues vary among the three main pathways of photosynthesis— $\text{C}_3$ ,  $\text{C}_4$  and CAM (Winter and Holtum, 2002) (Fig. 1)—with each carboxylation route creating a different fractionation. For example, a sugar from  $\text{C}_4$  sugarcane might have a  $\delta^{13}\text{C}$  of  $-10.5\text{‰}$ , as during carboxylation,  $^{13}\text{C}$  is assimilated more slowly than  $^{12}\text{C}$ . Sugar from beet, a  $\text{C}_3$  plant, typically shows a greater  $^{13}\text{C}$  depletion of  $-25\text{‰}$ .

### Stable isotopes in water

Isotope ratios of oxygen ( $\delta^{18}\text{O}$ ) and hydrogen ( $\delta^2\text{H}$  or  $\delta\text{D}$ ) in precipitation vary due to temperature, rainfall amount, elevation and distance from the coast (Dansgaard, 1953; Gourey *et al.*, 2005; Bowen, 2010). The isotopic composition of precipitation reflects fractionation processes during evaporation, transport, condensation and ultimately precipitation itself (Sprenger *et al.*, 2016). There is a global linear relationship between precipitation  $\delta^{18}\text{O}$  and  $\delta^2\text{H}$  first described by Craig (1961) as the Global Meteoric Water Line. Subsequent research investigated variations in Local Meteoric Water lines, which represent site-specific control on hydroclimatic processes (Rozanski *et al.*, 1993; Putman *et al.*, 2019). Precipitation that infiltrates into the soil and becomes plant-available water is further subject to fractionation caused by evaporation. Soil water generally becomes isotopically enriched (i.e. less negative) because of the preferential evaporation of the lighter isotopes (see Fig. 2, modified from Sprenger *et al.* (2016)). This effect varies by soil depth with generally

**Table 2:** Research questions selected from the 100 questions raised by Cooke *et al.* (2021) and 2 additional questions that are suitable for stable isotope approaches

| Question number (Cooke <i>et al.</i> , 2021) | Research question   | Research examples  | Isotopic analysis/indicator   | Example of expected isotopic response   |
|--|---|--|---|---|
| 1  | Can the potential for rapid evolution in physiological tolerance of threatened taxa be maximized?   | Analysis of physiological traits in hybrids and genotypes occurring on the edge of a species distribution      | $\delta^{13}\text{C}$ in leaf sugars, whole-leaf tissue, tree-ring cellulose  | Lower $\delta^{13}\text{C}$ in hybrids, reflecting lower stomatal sensitivity to soil water limitations and atmospheric drought   |
| 6  | How do interactions among plasticity, genetic drift and adaptation affect the resilience of populations to environmental change?  | Reciprocal common garden experiments conducted over multiple seasons and years                                 | $\delta^{13}\text{C}$ in leaf sugars, $\delta^2\text{H}$ , $\delta^{18}\text{O}$ in leaf and xylem water  | Lower $\delta^2\text{H}$ and $\delta^{18}\text{O}$ in xylem water of more xeric genotypes, reflecting deeper root systems to buffer against soil water limitations  |
| 9  | What physiological mechanisms determine the pace of thermal acclimation and adaptation?   | Analysis of stomatal behaviour and photosynthetic processes in response to temperature extremes                | $\delta^{13}\text{C}$ in leaf sugars and respired $\text{CO}_2$   | Lower $\delta^{13}\text{C}$ in plant taxa, reflecting lower stomatal sensitivity to thermal stress and atmospheric aridity  |
| 12   | How can physiological metrics (e.g. tree growth ring analysis, metabolic rate measures in ectotherms) provide long-term predictions of organismal sensitivity to global change? | Analysis of tree rings performed over multiple decades   | $\delta^{13}\text{C}$ , $\delta^{18}\text{O}$ in wood cellulose   | Lower inter-annual variability in tree-ring $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in tree taxa following drought, reflecting a lower long-term impact of drought on tree physiological performance                                      |
| 15   | How do changes in winter climate and snow and/or ice cover influence overwintering physiology of plants and animals?  | Analysis of plant water balance and drought stress over the growing season                                     | $\delta^{13}\text{C}$ , $\delta^{18}\text{O}$ in wood cellulose   | Lower inter-annual variability in tree-ring $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in tree taxa, reflecting lower drought stress following years of low winter precipitation/snowfall  |
| 17   | How will physiological systems adapt and respond to the interactive and cumulative effects of climate change?   | Comparison of physiological traits among populations occurring at the core and edges of a species distribution | $\delta^{13}\text{C}$ , $\delta^{18}\text{O}$ in whole-leaf tissue and/or tree-ring cellulose, $\delta^2\text{H}$ , $\delta^{18}\text{O}$ of xylem and soil water | Lower intra- and inter-annual variability in leaf or tree-ring $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in tree taxa occurring at the core relative to the edge of a species distribution, reflecting lower variability in stress exposure |
| 19   | To what extent does physiological resilience (or lack thereof) to environmental change of one target species affect or predict success of another?                              | Trait comparison of species with similar life history strategies in response to environmental gradients        | $\delta^{13}\text{C}$ in whole-leaf tissues, and/or tree-ring cellulose   | Similarities in temporal patterns of $\delta^{13}\text{C}$ among plant taxa with similar life history strategies  |
| 22   | How can physiological tools be best used to improve our capacity to monitor organismal and population responses to environmental change?  | Measurements of plant growth and leaf trait expression   | $\delta^{13}\text{C}$ in whole-leaf tissues, and/or tree-ring cellulose   | Increasing $\delta^{13}\text{C}$ over time, reflecting increased stress in plant taxa   |

Continued

Table 2: Continued.

| Question number (Cooke et al., 2021) | Research question   | Research examples  | Isotopic analysis/indicator   | Example of expected isotopic response   |
|--------------------------------------|---|--|---|---|
| 27                                   | How does human infrastructure and operations (e.g. hydropower, wind turbines, roads) affect the physiological status of wild organisms?                       | Comparison of organisms occurring at various distances from human infrastructure               | $\delta^{13}\text{C}$ , $\delta^{15}\text{N}$ of whole-leaf tissues, $\delta^2\text{H}$ , $\delta^{18}\text{O}$ of xylem water relative to soil water | Gradients in leaf $\delta^{13}\text{C}$ and/or $\delta^{15}\text{N}$ , increases in $\delta^2\text{H}$ and $\delta^{18}\text{O}$ of xylem water downstream of water impoundments, reflecting reduced soil water availability  |
| 32                                   | Which physiological attributes facilitate invasive species establishment and spread?  | Experiments to manipulate and quantify nutrient and water availability, N sources              | $\delta^{15}\text{N}$ of whole-leaf tissues, $\delta^2\text{H}$ , $\delta^{18}\text{O}$ of xylem water  | Higher $\delta^{15}\text{N}$ in leaf tissues, reflecting N sourced from local N pollution. Lower $\delta^2\text{H}$ and $\delta^{18}\text{O}$ of xylem water, reflecting high soil water availability   |
| 35                                   | Can physiological vulnerabilities in invasive species be identified and exploited to control them (i.e. know your enemy)?                                     | Experiments to examine Resource limitations and competition                                    | $\delta^{13}\text{C}$ of whole-leaf tissues, $\delta^2\text{H}$ , $\delta^{18}\text{O}$ of xylem water  | Contrasts of $\delta^{13}\text{C}$ between invasive plants and native plants, reflecting contrasts in response to resource limitations. Contrasts in xylem water $\delta^2\text{H}$ and $\delta^{18}\text{O}$ between invasive and native plants, reflecting contrasts in rooting depth |
| 41                                   | Are there physiological measures that can identify or inform tipping points of drought stress in plants, particularly threatened trees and shrubs?            | Comparison of tree rings in trees that survived drought versus those that succumbed to drought | $\delta^{13}\text{C}$ , $\delta^{18}\text{O}$ in wood cellulose   | Higher $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in cellulose of trees before drought in non-surviving trees relative to survivors, reflecting potential tipping points for predicting mortality of similar populations from future drought                                       |
| 44                                   | What are the physiological 'early warning signals' of population decline or collapse?   | Population-scale leaf trait analysis and/or tree-ring analysis                                 | $\delta^{13}\text{C}$ in whole-leaf tissue, $\delta^{13}\text{C}$ , $\delta^{18}\text{O}$ in wood cellulose   | Marked increases in $\delta^{13}\text{C}$ , $\delta^{18}\text{O}$ over time, reflecting temporal decreases in stomatal conductance and photosynthetic gas exchange  |
| 71                                   | Can physiological monitoring programs quantify the sublethal impacts of pollution and identify areas in most need of recovery efforts?                        | Tracing pollutants to their sources and the impact on plants                                   | $\delta^{15}\text{N}$ of plant tissues, such as leaves and roots  | Spatial gradients in tissue $\delta^{15}\text{N}$ along transects from pollution point sources, reflecting spatial gradients in pollution exposure  |
| 75                                   | Do organisms at the core and edges of populations differ in physiological phenotypes such that this can be exploited to enhance management?                   | Reciprocal common garden experiments placed at the core and edge of population distributions   | $\delta^{13}\text{C}$ in leaf sugars or whole-leaf tissue   | Lower $\delta^{13}\text{C}$ in populations on the warm edge of a species distribution, reflecting lower stomatal sensitivity to aridity   |
| 83                                   | How can candidate species for reintroduction be assessed to ensure that they are physiologically suited to the environment to which they would be introduced? | Measurements of leaf gas exchange, leaf trait expression and water source determination        | $\delta^{13}\text{C}$ , $\delta^{15}\text{N}$ of whole-leaf tissue, $\delta^2\text{H}$ , $\delta^{18}\text{O}$ of xylem water                         | Matching $\delta^2\text{H}$ and $\delta^{18}\text{O}$ between xylem water and groundwater or other stable water source, reflecting the establishment of deep root systems   |

Continued



**Table 2:** Continued.

| Question number (Cooke <i>et al.</i> , 2021) | Research question   | Research examples   | Isotopic analysis/indicator   | Example of expected isotopic response   |
|--|---|---|---|---|
| 87   | Which physiological functions in plants can be used to select candidates to restore landscapes following disturbances?  | Measurements of growth rates and stress in plants reintroduced following disturbance                        | $\delta^{13}\text{C}$ in leaf sugars, respiration and whole-leaf tissue   | Low values of leaf $\delta^{13}\text{C}$ following re-establishment of disturbed habitats, reflecting acclimation to disturbed conditions   |
| 98   | What methods best predict which species will respond well to urban environments on the basis of physiological traits, compared with those that do poorly, regardless of their rarity in natural ecosystems? | Measurements of plant responses to enhanced night-time temperatures and ozone exposure                      | $\delta^{13}\text{C}$ in leaf sugars, respiration and whole-leaf tissue, $\delta^{13}\text{C}$ , $\delta^{18}\text{O}$ in tree-ring cellulose | Low values of leaf $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in tree-ring cellulose in trees established in urban habitats, reflecting acclimation to urban environmental conditions. |
| Additional question 1                        | What physiological tools can be used to identify and combat the illegal harvest of threatened and endangered species?   | Forensic approaches to identify source location of illegally traded specimens                               | $\delta^{18}\text{O}$ in whole-plant tissues or cellulose   | Matching between $\delta^{18}\text{O}$ of source waters and modelled $\delta^{18}\text{O}$ of xylem water   |
| Additional question 2                        | What are the best practices to monitor and manage plant–microbe symbioses in habitats impacted by disturbance and climate change?   | Identify the presence of fungal mutualists following large-scale restoration projects following disturbance | $\delta^{13}\text{C}$ in soil organic matter and soil respiration   | Reduced $\delta^{13}\text{C}$ in soil organic matter, reflecting allocation of sugars from canopy-pulse-labelled $\text{CO}_2$  |

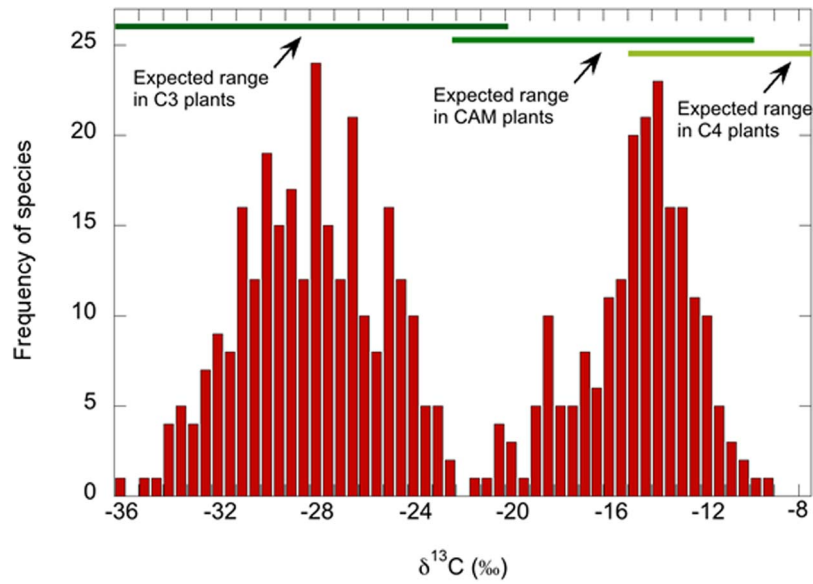
**Table 3:** Definitions of key terms used throughout the paper

| Term                   | Definition  |
|------------------------|---|
| Isotopic fractionation | The relative partitioning of heavier and lighter isotopes between two coexisting phases (e.g. water in the liquid and vapour phases)    |
| Isotopic enrichment    | Isotopic fractionation that increases the abundance of the heavier isotope relative to the lighter isotope                              |
| Water-use efficiency   | The amount of photosynthetic carbon gain per unit transpirational water loss or stomatal conductance                                    |
| Isotopic mixing models | Models that calculate the contributions of different isotopic sources in a sample   |
| Isoscapes              | Spatially explicit predictions of isotope ratios that are produced from process-based models  |
| Isotope chronologies   | A time series of stable isotope signatures, typically (for plants) measured in tree growth rings, cactus spines and moss or peat layers |

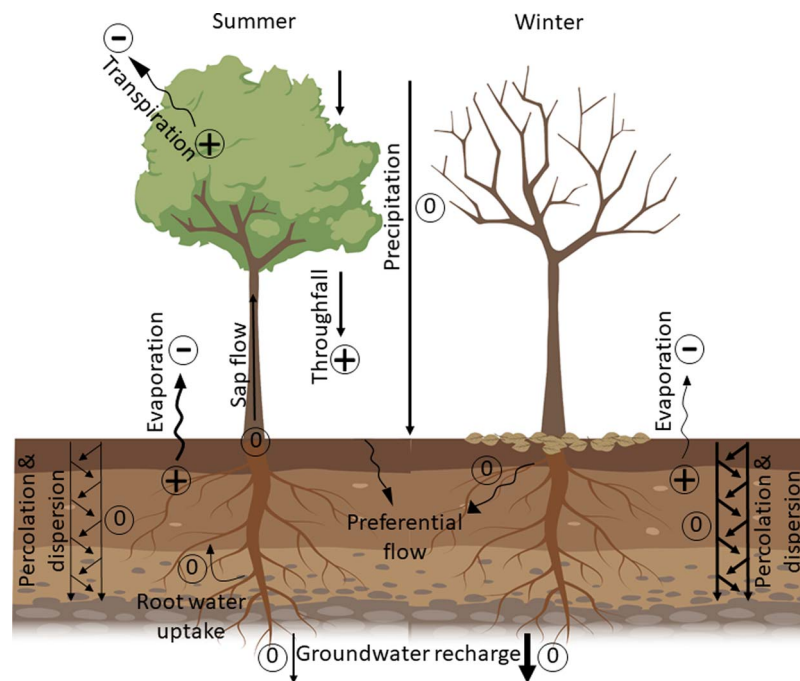
more enrichment of shallow soil water and less with depth due to evaporative processes in shallow soils (Ehleringer and Dawson, 1992; Dawson and Ehleringer, 1998). Furthermore, groundwater that was recharged at higher elevation or during cool winter months may be more similar to precipitation and less enriched, leading to isotopic variation within the soil profile and capillary fringe associated with groundwater. Because plants generally do not substantially fractionate water through root uptake and xylem transport to the canopy, xylem water can be used to determine where within the soil profile plants are obtaining their water (see Cernusak *et al.*, 2016; Ehleringer and Dawson, 1992). Although, this may not be applicable in all habitats, such as dry or saline habitats

(Lin and Sternberg, 1993; Ellsworth and Williams, 2007), wet-dry or even mesic habitats (Evaristo *et al.*, 2017; Barbeta *et al.*, 2019; Vega-Grau *et al.*, 2021).

Similar to water in shallow soils, water in leaves is subject to evaporative enrichment during transpiration as stomata open to acquire  $\text{CO}_2$  and/or cool leaves (Cernusak *et al.*, 2016). The amount that leaf water is enriched relative to xylem water depends primarily on the leaf-to-air vapour pressure gradient and leaf water turnover rates that are governed by leaf morphology (e.g. leaf size, vein structure) and stomatal regulation of leaf water fluxes. The enriched leaf water is ultimately used in photosynthesis to construct



**Figure 1:** The  $\delta^{13}\text{C}$  values recorded from 506 different plant species from nine families. Note that CAM species are relatively small in number (mainly restricted to xeric habitats) and are rather similar in their metabolism and fractionations to  $\text{C}_4$  plants. The data were redrawn from Winter and Holtum (2002).



**Figure 2:** Redrawn from Sprenger *et al.* (2016). Depiction of the processes affecting the pore water stable isotope composition in the vadose zone during summer and winter in a temperate climate. The plus sign indicates an isotopic fractionation process leading to enrichment in heavy isotopes; the minus sign represents depletion in heavy isotopes; and the zero is a sign of non-fractionating processes. However, during water uptake fractionation may occur in some root systems, especially at the root tips. The text indicates the labels of the closest two arrows. Detailed information about spatiotemporal variations of each process are given in Sprenger *et al.* (2016).

sugars from  $\text{CO}_2$  that diffuses into the leaf (Farquhar *et al.*, 1998; Farquhar *et al.*, 2007). Thus, the  $\delta^{18}\text{O}$  in sugars used

to build plant tissues reflects the isotopic composition of soil water taken up by the roots and the amount of evaporative

enrichment of leaf water during transpiration, plus post-photosynthetic exchange (Yakir and DeNiro, 1990; Roden *et al.*, 2000; Barbour *et al.*, 2004).

### Carbon isotopes in plant tissues

Within a given photosynthetic pathway,  $\delta^{13}\text{C}$  in plant tissues mainly reflect the balance between the supply of  $\text{CO}_2$  into a leaf, controlled by stomatal conductance, and the demand for  $\text{CO}_2$  by photosynthetic enzymes (Fig. 3a; Farquhar *et al.*, 1989). For  $\text{C}_3$  plants,  $\delta^{13}\text{C}$  ranges from  $-35\text{‰}$  to  $-20\text{‰}$ ; for  $\text{C}_4$  plants, from  $-15\text{‰}$  to  $-7\text{‰}$ ; and for CAM plants, from  $-22\text{‰}$  to  $-10\text{‰}$  (Osmond *et al.*, 1982; Ehleringer, 1989; Ehleringer and Osmond, 1989). For  $\text{C}_3$  plants,  $\delta^{13}\text{C}$  values are a reasonable proxy of water-use efficiency (WUE), with more enriched (less negative)  $\delta^{13}\text{C}$  values indicating higher WUE and lower  $\delta^{13}\text{C}$  indicating lower WUE (Farquhar *et al.*, 1982). WUE is often defined as the ratio of photosynthesis ( $A$ , assimilation) over stomatal conductance ( $g_s$ ), which reflects the supply of  $\text{CO}_2$  relative to the demand by the photosynthetic enzymes (with carbon fixation accompanied by kinetic fractionation). Thus,  $\delta^{13}\text{C}$  of leaf tissue and tree rings can be used to assess changes in WUE, in response to soil water availability, heatwaves and other stressors (e.g. van der Sleen *et al.*, 2017). Furthermore, measuring  $\delta^{18}\text{O}$  in tree rings can help resolve whether variation in  $\delta^{13}\text{C}$  was due to changes in stomatal conductance or changes in photosynthetic capacity (Shestakova *et al.*, 2014; de Boer *et al.*, 2019). The  $\delta^{18}\text{O}$  of cellulose should reflect differences in stomatal conductance, vapour pressure deficit (VPD) and transpiration, which lead to differences in evaporative fractionation in leaves that would then be recorded in tree-ring cellulose (Barbour *et al.*, 2004; Barbour, 2007; de Boer *et al.*, 2019). This dual isotope approach produces a general relationship for  $\text{C}_3$  plants, where a positive relationship between  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  generally indicates that changes in  $\delta^{13}\text{C}$  are due to larger changes in stomatal conductance relative to photosynthetic capacity, while a negative relationship indicates larger changes to photosynthetic capacity relative to stomatal conductance (Scheidegger *et al.*, 2000; Barnard *et al.*, 2012). However, studies of this dual isotope approach have had varied results due to uncertainties in the interpretation of  $\delta^{18}\text{O}$  that arise from variation in source water and atmospheric  $\delta^{18}\text{O}$  and species-specific differences in the effect of relative humidity on stomatal conductance. Therefore, these studies should be interpreted with caution (for details, see Roden and Siegwolf, 2012); Roden and Farquhar, 2012).

### Nitrogen isotopes in plant tissues

Applications of stable nitrogen (N) isotopes center on N sources (reviewed by Marshall *et al.*, 2007), with  $\delta^{15}\text{N}$  considered both as an integrator of the N cycle and plant stress responses (reviewed by Robinson, 2001). Plant tissues and xylem water can be depleted or enriched relative to atmospheric  $\text{N}_2$  (defined as  $0\text{‰}$ ). A  $\sim 3\text{‰}$  isotopic enrichment with each trophic level (Minagawa and Wada, 1984; Perkins *et al.*,

2014) causes distinct  $\delta^{15}\text{N}$  signatures in plants that access N from different trophic levels. Plants relying on biologically fixed  $\text{N}_2$  can mirror atmospheric  $\text{N}_2$  (Shearer *et al.*, 1983), while plants relying on soil N acquire enriched N because the transformations in soil (e.g. microbial denitrification) cause the loss of isotopically light N. However, a strict dichotomy of  $\delta^{15}\text{N}$  of  $\text{N}_2$ -fixing and non-fixing plants is often not observed as plant signatures reflect root specializations and N source preferences (Robinson, 2001; Marshall *et al.*, 2007) with numerous processes influencing  $\delta^{15}\text{N}$ : discrimination against  $^{15}\text{N}$  during uptake, mycorrhizal fungi passing on isotopically light N, rooting depth and isotopically distinct N sources (e.g. isotopically enriched ammonium compared with nitrate). Like other stable isotope pairs, plant parts can differ in  $\delta^{15}\text{N}$ . For example,  $\delta^{15}\text{N}$  of root xylem water emerged as a more direct indicator of biological  $\text{N}_2$ -fixation than foliar  $\delta^{15}\text{N}$ , which overlapped in  $\text{N}_2$ -fixing and non-fixing trees (Soper *et al.*, 2015b). As succinctly put by Robinson (2001), the ‘information derived from  $\delta^{15}\text{N}$  is often circumstantial rather than definitive.  $\delta^{15}\text{N}$  can provide clues about, or reveal the ‘footprint’ of a process, but not necessarily deliver conclusive evidence for its cause’.  $\delta^{15}\text{N}$  signatures when carefully applied, can show robust trends ideal to applications in conservation.

## Water isotopes and resource acquisition

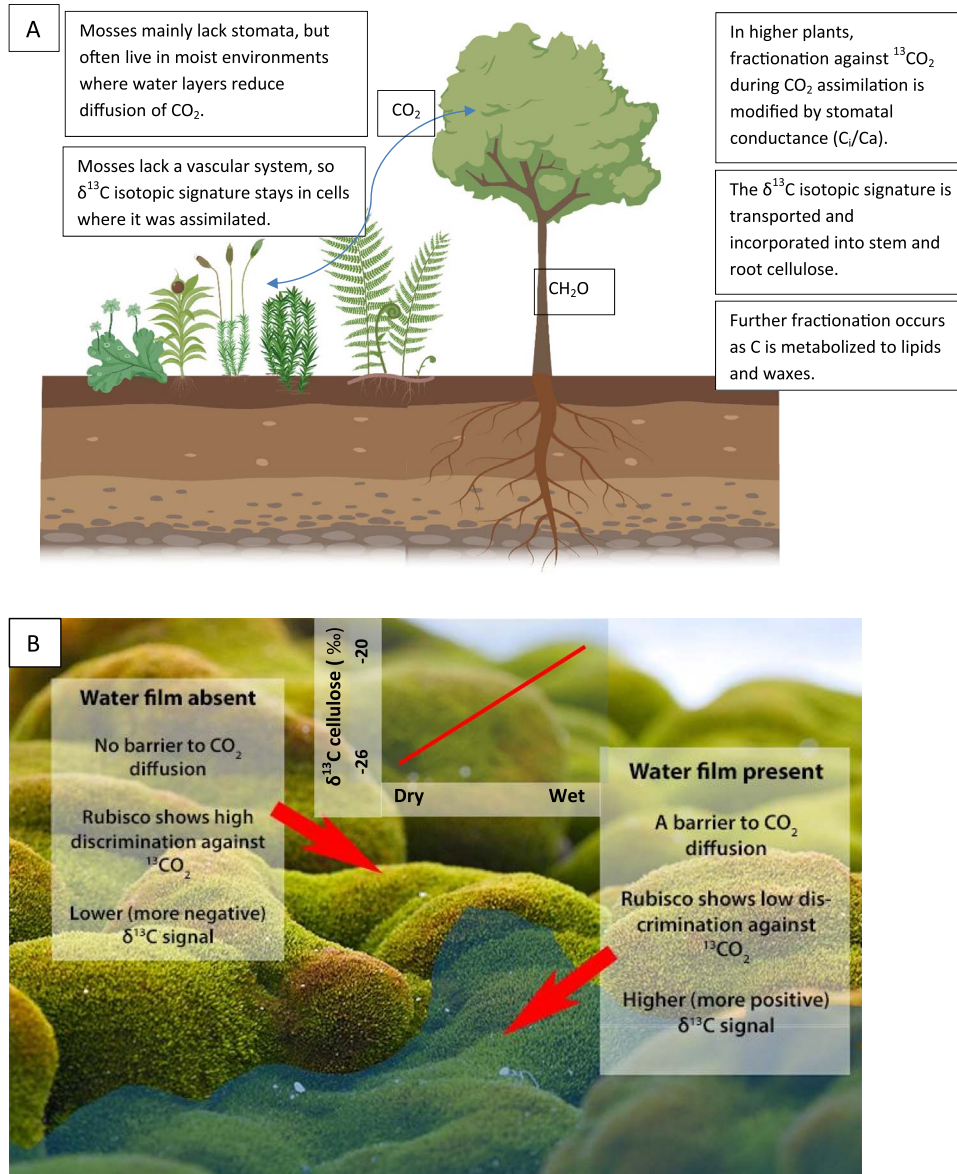
### Short-term $\delta^{18}\text{O}$ and $\delta^2\text{H}$

#### Conservation of riparian tree species and groundwater-dependent systems

Stable isotopes of oxygen ( $\delta^{18}\text{O}$ ) and hydrogen ( $\delta^2\text{H}$ ) can be used to determine plant water sources by comparing the stable isotopic values of plant xylem water to the stable isotopic values of all potential source waters (Ehleringer and Dawson, 1992). Water in shallow soils is often more isotopically enriched by evaporative fractionation than deep soil water, stream water and groundwater. Using isotopic mixing models, the fractional contribution and its associated probability can be determined for each water source (Phillips and Gregg, 2003; Evaristo *et al.*, 2017). These studies generally are short-term and track plant water use through a growing season or several growing seasons, by sampling plants at various times throughout the year. Short-term  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  surveys provide snapshots of plant water use in response to drought, heatwaves or other stressors, such as human alterations to river flows or declining groundwater levels (Flanagan *et al.*, 2019). These studies can aid conservation and restoration practitioners on the proper selection of species and genotypes and/or necessary modifications to restore hydrologic function of ecosystems such as riparian or wetland systems (Schook *et al.*, 2020) (e.g. Question 27 in Cooke *et al.*, 2021; Table 2).

One of the most ecologically important and threatened forest types is riparian forests that are often characterized by a





**Figure 3:** Schematic representation of physiological processes that determine carbon isotopic signatures in higher plants and mosses (A). Fractionation against  $^{13}\text{C}$  occurs during diffusion through stomatal pores in leaves, or water layers over mosses (B), and assimilation by Rubisco. In higher plants, assimilates are transported and incorporated into other parts of the plants. This does not happen in plants that lack a vascular system, including most mosses. Downstream modification can also occur, e.g. to create lipids or waxes. Lower panel. (B) shows how a water layer over mosses creates a barrier to diffusion of  $\text{CO}_2$ . This means that moss  $\delta^{13}\text{C}$  can be used as a proxy for water availability in the moss's micro-environment. For example,  $\delta^{13}\text{C}$  in samples of the Antarctic moss, *C. purpureus*, reveal a positive  $\delta^{13}\text{C}$  gradient with wetness; from dry (more negative) at the top of the slope to wet (more positive) at the lake's edge (inset graph, sketch from Bramley-Alves *et al.*, 2015). Photo reproduced with permission from Jessica Bramley-Alves.

mix of obligate phreatophytes (deep-rooted plants) and facultative phreatophytes. Obligate phreatophytes rely on growing their roots to the capillary fringe above groundwater, while facultative phreatophytes can use both shallow and deep roots to obtain water for transpiration. Alteration of hydrologic regimes due to dams, groundwater pumping and water diver-

sions can alter the availability of streamflow and groundwater, while weather variability and changing climate can alter the availability of precipitation. Successful restoration efforts thus require knowledge of the various dependencies of riparian species on these different water sources. For example, in the southwestern United States, Fremont cottonwood

(*Populus fremontii*) and Goodding's willow (*Salix gooddingii*) are important components of riparian systems (Stromberg, 2001). Analyses of  $\delta^{18}\text{O}$  of xylem water determined that *P. fremontii* occurring along the San Pedro River—one of the few free-flowing rivers in the western United States—relied on groundwater during dry periods but could opportunistically use shallow soil water from monsoon rains. Alternatively, *S. gooddingii* was strictly deep-rooted and relied exclusively on groundwater throughout the growing season (Snyder and Williams, 2000). Another study on the riparian species box elder (*Acer negundo*) found a similar opportunistic use of rainwater during rainy periods and reliance on groundwater during dry periods (Kolb *et al.*, 1997). There is also evidence for a strong genetic component in root distribution patterns within phreatophytic species that may be detected with stable-isotope analysis (Hultine *et al.*, 2020b). For example, *P. fremontii* genotypes sourced from intermittent streams produce deeper roots than genotypes sourced from perennial reaches (Hultine *et al.*, 2020a). Rooting depth and root elongation rates may be detected experimentally with stable-isotope analysis as trees extend roots through soil profiles with contrasting water isotopic signatures. Thus, the use of stable isotopes in water has the potential to impact riparian restoration by uncovering rooting habits and allow practitioners to match genotypes with predicted future hydrological conditions (e.g. Questions 17, 22 in Cooke *et al.*, 2021; Table 2).

### Woody plant responses to ecological drought in dryland regions

Vegetation in drylands is particularly vulnerable to climate change because soil moisture may become increasingly limited as temperatures and potential evapotranspiration increase (IPCC, 2014; USGCRP, 2017). Ecohydrological simulation models indicate that feedbacks between climate change and vegetation change will exacerbate soil water limitations in most dryland regions over the next century (Schlaepfer *et al.*, 2017; Tietjen *et al.*, 2017). Stable isotopes in water may be a powerful tool to identify woody plant species that are most resilient to the effects of ecological droughts. In turn, resilient plant assemblages may limit the synergistic impacts of climate change and vegetation change on ecological drought.

In the deserts of the United States as well as in other ecosystems, summer and winter rainfall can have distinct isotopic signatures due to differences in the formation and precipitation history of rainfall during the growing season in comparison with precipitation during the winter. These signatures have been valuable in understanding how plant species react to changes in climate that affect the seasonality of precipitation (White *et al.*, 1985; Ehleringer *et al.*, 1991; Donovan and Ehleringer, 1994; Phillips and Ehleringer, 1995; Dawson and Ehleringer, 1998). Williams and Ehleringer (2000) studied three dominant woody species along a gradient of increasing monsoon rainfall and sampled  $\delta^{18}\text{O}$  and  $\delta^2\text{H}$  over two growing seasons. Gambel oak (*Quercus gambelii*) used only deep soil water along the monsoon gradient, while

two-needle pinyon (*Pinus edulis*) and Utah juniper (*Juniperus osteosperma*) used a large proportion of shallow soil water after monsoon rain events highlighting species-specific responses to changes in the seasonality of precipitation. Coupled with other measurements of plant growth and stress, stable isotopes can be used to elucidate the responses of species to changing environmental conditions. Furthermore, in the above-mentioned study, irrigation with deuterium-labelled water suggested that, for *P. edulis*, high soil temperatures may limit activity of shallow roots, thus reducing the ability of some shallow-rooted species to use monsoon rainfall (Williams and Ehleringer, 2000). Increasing soil temperature highlights the need to identify species and genotypes that can sustain roots over a long-enough duration to forage for soil moisture, particularly at the seedling stage of development. Coupling stable isotopic measurements in water with ecohydrological simulation modelling is a potentially robust approach for identifying resilient plant traits in a changing climate (Brinkmann *et al.*, 2018) (e.g. Question 17 in Cooke *et al.*, 2021; Table 2).

### Longer-term studies of water isotopes

#### Using Iso-forensics for plant conservation

Stable isotopes have been used in forensic applications to isolate the provenances (origins) of various plant materials. This has helped determine the potential geographic sources of counterfeit currency, plant-derived illegal drugs, adulterated food and beverages, sources of explosives and even to identify the origins of human remains (Ehleringer *et al.*, 1999; Ehleringer *et al.*, 2008; Bartelink *et al.*, 2016). The origins of forensic research efforts were based in ecological studies to understand hydrological inputs, plant water sources, animal migration patterns and the relation of flora and fauna to climate (Gat and Gouffiantini, 1981; Kelly *et al.*, 1991; Ehleringer and Dawson, 1992; Comstock and Ehleringer, 1992a; Hobson, 1999; McGuire and McDonnell, 2007). Spatially explicit maps were created by evaluating the natural abundance of stable isotopes over ecological and geological systems to generate predictive models that incorporate spatial variables to construct isotope landscapes, also known as isoscapes (Bowen, 2010). For example, the stable isotopic composition of precipitation has been used to identify the provenance of plant products (West *et al.*, 2007b). Likewise, an isoscape of the composition of tap water has been used to identify regions of possible habitation for missing persons using tooth enamel and hair from human remains (Bartelink *et al.*, 2016).

There is a potential to use the isoscape framework to target illegal trade of rare plants, which leads to an additional conservation question (Table 2): what physiological tools can be used to identify and combat the illegal harvest of threatened and endangered species? For example, one potential, yet untested, application of the isoscape framework is confronting the illegal international trade of endangered cactus plants. Cacti are among the most threatened taxonomic

groups on the planet with over 30% of all species of cacti considered threatened or endangered (Goettsch *et al.*, 2015; Hultine *et al.*, 2016b). The illegal trade of cacti is recognized as a primary contributor to the endangered status of this taxon (Goettsch *et al.*, 2015; Hultine *et al.*, 2016a). Because many cactus species are endemic to small areas, their tissues should only record a small range of values of  $\delta^{18}\text{O}$  and  $\delta^2\text{H}$  in the wild and should be predictable using isoscape modelling approaches (West *et al.*, 2007b). Thus, stable isotopes of cacti tissues, such as spines, could be analysed from plants obtained from points of entry along international borders to detect whether plants are collected illegally from wild populations or cultivated legally in greenhouse facilities. However, using isoscapes to identify illegally traded cacti needs further refinement. Water cycling in cactus stems, particularly giant cacti such as giant saguaro (*Carnegiea gigantea*) can be slow with water turnover rates of potentially more than a year, leading to large seasonal and inter-annual swings in tissue stable-isotope values (English *et al.*, 2007; Hultine *et al.*, 2019). These large swings limit the capacity for iso-forensic applications to pinpoint a source location for an individual plant, and thus could limit the capacity for these data to stand up under legal scrutiny for giant cacti. Nevertheless, isotopic variation in small-stemmed species of cacti—such as those that are primarily subjected to illegal trading—is likely much smaller than that reported in giant cactus species. Consequently, there is considerable potential to use isoscape approaches as a much-needed tool to combat the illegal trade of this ecologically important plant family (e.g. Additional Question 1, Table 2).

### Limitations and on-going questions in water isotope research

Although water isotopes have been used to determine water sources, it is still a field of active research. Plant xylem water in dual isotope space ( $\delta^2\text{H}$  and  $\delta^{18}\text{O}$ ) can be more evaporatively enriched than rain, snow melt, stream water, groundwater and even soil water (Brooks *et al.*, 2010; Goldsmith *et al.*, 2012; McDonnell, 2014; Evaristo *et al.*, 2015; Bowling *et al.*, 2017; Berry *et al.*, 2018; Penna *et al.*, 2018). The observed phenomenon whereby plant xylem water is isotopically distinct from water inputs that rapidly recharge streamflow and groundwater (Brooks *et al.*, 2010; Goldsmith *et al.*, 2012; McDonnell, 2014; Evaristo *et al.*, 2015) has been attributed to a fast-moving pool of ‘mobile waters’ versus an ‘immobile’ pool of plant-available water that is tightly bound in soil pores. These observations challenged previous assumptions that soil water was well mixed following precipitation events or that new water uniformly displaced antecedent soil water deeper into the soil profile (Brooks *et al.*, 2010; Sprenger and Allen, 2020). In some cases, xylem water was isotopically more evaporatively enriched than the soil moisture evaporation line (Evaristo *et al.*, 2015; Evaristo *et al.*, 2016), highlighting that other processes deserve further consideration. Potential processes include the following: fractionation within

the plant that could be more widespread than expected due to water exchanges at the Casparian strip and mycorrhiza that may be involved in water uptake; plant embolisms that change liquid water to vapour within the xylem; and the transfer of evaporatively enriched phloem water to xylem water (Berry *et al.*, 2018). A review (von Freyberg *et al.* (2020) suggests ways to move forward including designing experiments that (i) take advantage of extreme variation in water sources, (ii) take advantage of sampling precipitation after extremely dry periods, (iii) use labelling experiments (see section on labelling below), (iv) use potted plant experiments and (v) increase the temporal and spatial resolution of isotopic sampling.

## Carbon isotopes and resource acquisition

### Short-term carbon isotope ratios

Stable carbon isotopic composition of fully developed leaves in  $\text{C}_3$  plants generally reflect their WUE. Therefore, measurement of  $\delta^{13}\text{C}$  of leaves to determine WUE can be useful to assess the following: which plant species or genotypes can respond to changes in precipitation patterns and rising temperature; plant materials suitable for restoration of functionally changed ecosystems; and plant species or genotypes suitable for assisted migration. The ability of plants to survive increased temperatures and extended periods of drought is predicted to govern changes in species ranges as a consequence of climate change (Wilson *et al.*, 2005; Vitt *et al.*, 2010; Crimmins *et al.*, 2011; Pauli *et al.*, 2012). In a review of key traits for assessing adaptive phenotypic plasticity across a range of species, WUE assessed through stable isotopes was 1 of 11 key traits identified (Nicotra *et al.*, 2010). The effects of climate change may be amplified in high elevation hotspots of biodiversity (IPCC, 2013), which are often characterized by rare and endemic species (Casazza *et al.*, 2005; Casazza *et al.*, 2008). However, widely distributed species that span a range of temperature and moisture gradients and are critically important for regional productivity are also at risk depending on the ability of these populations to adapt to increased temperatures and drought. We provide two examples, a rare endemic species and widely distributed forest species, to illustrate how stable isotopes can guide management decisions regarding conservation. *Heterotheca brandegeei* is a perennial pincushion plant endemic to rocky high elevation outcrops in Baja California, Mexico. Winkler *et al.* (2020) used growth chambers to determine the effects of drought and predicted temperature increases on the early life stage responses and phenotypic plasticity of *H. brandegeei*. Leaf-level WUE, measured using the  $\delta^{13}\text{C}$  of leaves, did not change in response to warming or drought alone; however, drought in combination with warming significantly increased WUE. This increase in WUE was partly explained by maternal lineage, indicating a role of genetic variance in adaptive plasticity that is detectable



using measurements of leaf  $\delta^{13}\text{C}$  ratio (e.g. Questions 6 and 17 in Cooke *et al.*, 2021; Table 2).

Aleppo pine (*Pinus halepensis*) is the most common coniferous tree in the Mediterranean and spans a wide range of temperature and moisture gradients. Intraspecific genetic differentiation in the various climatic regions has been proposed as likely to shape this species' response to the magnitude and timing of droughts (Voltas *et al.*, 2008). Voltas *et al.* (2008) used a common garden experiment at two sites with 25 *P. halepensis* populations that spanned the species' geographic range. They analysed the  $\delta^{18}\text{O}$  and  $\delta^{13}\text{C}$  of hemicellulose in tree rings of 3-year-old *P. halepensis* branches. Trees from moist regions had lower WUE and grew more rapidly, while trees from dry regions had greater WUE and grew more slowly. The positive relationship between  $\delta^{18}\text{O}$  and  $\delta^{13}\text{C}$  for *P. halepensis* indicated that variation in  $\delta^{13}\text{C}$  was mainly driven by differences in stomatal regulation. Specifically, VPD, the length of seasonal drought and total rainfall were climate characteristics that influenced WUE. Measuring how WUE shifts in response to changes in climate is a potentially powerful tool for guiding successful conservation and restoration success of rare/endemic and widespread species (e.g. Questions 17, 22 and 75 in Cooke *et al.*, 2021; Table 2).

WUE and its interpretation is a key concept in plant ecology with debate as to whether high or low WUE confers a fitness advantage because it is highly dependent on the timing and severity of water stress (Heschel and Riginos, 2005) and may vary contextually by biome (see review by Nicotra and Davidson, 2010). In the case of the endemic perennial pincushion example, higher WUE coupled with mortality in the most severe treatments was indicative of high WUE reflecting greater stress and not greater fitness. In dry environments, selection favouring high WUE was sometimes associated with increased fitness, while other times lower WUE was associated with increased fitness and, in some cases, there was no correlation between WUE and fitness (Nicotra and Davidson, 2010). These results indicate that there may be opportunities to use  $\delta^{13}\text{C}$  measurements as proxies of WUE to predict fitness of individual plants before they are planted *ex situ* to restore plant populations and communities.

There are complexities and potential pitfalls with  $\delta^{13}\text{C}$  analysis of leaves that need to be considered. For example, in some species there are differences in young versus old leaves—with young leaves switching from being dependent (heterotrophic) on other leaves or carbohydrate storage until photosynthetic capacity develops in the new leaf (autotrophic) (Cernusak, 2020). The stable carbon isotope ratio of some species may also reflect the environment when the leaves were formed and not the entire growing season, and for some species this reflects periods of high water availability in the spring during leaf flushes (Comstock and Ehleringer, 1992a,b; Graham *et al.*, 2014). For some deciduous species, the growth of new leaves relies upon stored carbohydrates fixed during the previous growing season, thus creating time

lags between the current resource environment and past environmental conditions (Ehleringer *et al.*, 1992). Therefore, the interpretation of leaf  $\delta^{13}\text{C}$  is not straightforward because the timing of leaf formation relative to the acquisition of carbon is not constant. One way to reduce temporal lags between leaf formation and current conditions is to measure leaf photosynthates (i.e. soluble sugars) that usually have a 24–72-hour turnover rate (Brugnoli *et al.*, 1988; Fravolini *et al.*, 2005; Hultine *et al.*, 2013). However, leaf soluble sugar extraction is fairly labourious compared with analysing whole-leaf tissue.

Additional challenges arise from the variation in leaf  $\delta^{13}\text{C}$  that occurs at a variety of scales within leaves, within individual plants, within a species, among species and among species across climatic gradients (for global review, see Cornwell *et al.*, 2018). This variation has been attributed to variation in stomatal aperture (Farquhar *et al.*, 1982; Farquhar and Richards, 1984), the photosynthetic biochemistry of a leaf (Virgona *et al.*, 1990) and the difference in diffusion of  $\text{CO}_2$  within the mesophyll (Evans *et al.*, 1986; Barbour *et al.*, 2010). Fractionation can continue to occur as more complex compounds are produced within plants (Fig. 3a). For example, fractionation during the formation of lipids and waxes means that gradients in  $\delta^{13}\text{C}$  from the epidermis to the leaf center can be considerable, e.g. up to 4.3‰ in CAM plants (Robinson *et al.*, 1993). Importantly, variation in mesophyll conductance can at least partially decouple measurements of leaf  $\delta^{13}\text{C}$  from WUE. For example, mesophyll conductance can vary substantially across a broad range of species and result in up to a 3-fold difference in carbon isotope discrimination among species (Warren and Adams, 2006). This means that care must be taken to compare similar tissue types and in making inferences from leaf  $\delta^{13}\text{C}$ .

### Long-term responses of tree-ring stable-isotope ratios $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ —the need for climate proxies

Tree-ring widths from a composite of several trees have been used by dendrochronologists to successfully reconstruct past climates (Cook *et al.*, 2010). Stable isotopes of oxygen and carbon in tree rings have also been used to infer past climatic variables such as temperature, relative humidity, the occurrence of drought and tree physiological responses to droughts and soil moisture (Saurer *et al.*, 1997; McCarroll and Loader, 2004; Sarris *et al.*, 2013; Lavergne *et al.*, 2017; van der Sleen *et al.*, 2017). The processes that affect the stable-isotope ratios  $\delta^{18}\text{O}$  and  $\delta^{13}\text{C}$  in tree rings include canopy temperature, transpiration and photosynthetic uptake of  $\text{CO}_2$ . These processes are directly linked to meteorological variables such as air temperature, VPD, solar irradiance and available soil moisture from precipitation, which make stable isotopes of tree rings useful to evaluate species-specific physiological responses, as well as useful proxies of past climates (Hartl-Meier *et al.*, 2015). However, the biophysical processes that regulate the

synthesis of sugars and starches, transport processes between phloem and xylem and the ultimate conversion of photosynthate into lignin and cellulose in tree rings are complex and still not completely resolved, in particular regarding post-photosynthetic fractionations. These fractionation processes can decouple leaf processes recorded in stable-isotope ratios of transpiration and photosynthesis from the stable-isotope ratios recorded in tree rings, though much progress has been made (see review by Gessler *et al.*, 2014).

Furthermore, tree-specific strategies of adjusting individual physiologies can be revealed in stable isotopes of tree rings such as long-term responses to climate (McCarroll and Loader, 2004; van der Sleen *et al.*, 2017). Longer-term analyses of  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  in different tree genotypes can provide important information on mitigating the effects of extreme drought by identifying drought-resistant genotypes. Additionally, surveys of stable-isotope chronologies in a forest experiencing mortality can yield predictive tools to assess where mortality may be greatest and identify priorities for restoration planning (Maier *et al.*, 2019). For example, Schook *et al.* (2020) compared the tree-ring width and  $\delta^{13}\text{C}$  of riparian cottonwoods (*Populus angustifolia* and *P. angustifolia*  $\times$  *P. trichocarpa*) along a partially dewatered river reach to watered reaches over a 50-year time series. Tree-ring width took decades to respond to lowered water availability, while  $\delta^{13}\text{C}$  reflected dewatering immediately. Thus,  $\delta^{13}\text{C}$  provided an early indication of branch and tree mortality (see also Rood *et al.*, 2013) (e.g. Questions 12, 41 and 44 in Cooke *et al.*, 2021; Table 2).

Stable isotopes in tree rings and other long-lived plant tissues such as cactus spines (and even bryophytes; see below) have the potential to be powerful tools in conservation physiology. The vertically arranged chronological series of cactus spines provides a non-invasive, sensitive and high-resolution time-series of the physiological and metabolic responses of cacti plants to environmental variability that are similar to tree-ring isotope records (English *et al.*, 2007; Hultine *et al.*, 2019). For example, a 58-year-old  $\delta^{18}\text{O}$  spine chronosequence in the threatened and long-lived giant cactus, cardón (*Echinopsis atacamensis*), on the Bolivian Altiplano revealed varying degrees of plant water evaporation and stem recharge over time (English *et al.*, 2021). Moreover, minimum annual  $\delta^{18}\text{O}$  in spines had a strong positive relationship to the annual mean of minimum monthly temperatures (i.e. lower annual mean minimum temperatures lead to lower VPD, lower evaporation and lower  $\delta^{18}\text{O}$  values in spines). These results show that during the cool/cold night temperatures of the Altiplano (4000 masl), small increases in temperature can have an exponential effect on VPD, leading to higher  $\delta^{18}\text{O}$  in spines (i.e. more evaporated cactus stem water) in years with higher minimum (night-time) temperatures. Thus, spine  $\delta^{18}\text{O}$  chronologies could reveal changes in temperature that may impact the water balance of this culturally important yet threatened cactus species (e.g. Questions 9, 12, 15 and 17 in Cooke *et al.*, 2021; Table 2).

## Long-term responses of bryophyte stable-isotope ratios $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$

In addition to these examples of large woody plants, stable isotopes have been used to better understand the response of some of the smallest, non-vascular plants to climate and landscape change. Some mosses contribute to the development of peat banks, which record thousands of years of climate history and in which both carbon and oxygen isotopes are used to infer changes in temperature, water availability and water sources over millennia (Björck *et al.*, 1991; Royles *et al.*, 2012; Royles *et al.*, 2013a; Royles *et al.*, 2013b; Royles and Griffiths, 2015). Recently, stable isotopes have also been applied to living shoots of long-lived (>100 years) moss plants, providing information on recent changes in local environments (Clarke *et al.*, 2012; Robinson *et al.*, 2018). Such data can be particularly useful in alpine and polar regions where mosses tend to be dominant components of the flora and to sequester considerable amounts of carbon.

In Antarctica, stable isotopes of carbon have been shown to reflect the extent to which mosses are growing in water-saturated or dry, exposed microclimates (Fig. 3b). As non-vascular plants with reduced movement of assimilates between cells and tissues, many mosses lay down cellulose in sequential layers (similar to vertical tree rings; Clarke *et al.*, 2012; Robinson *et al.*, 2018). Because mosses lack stomata, isotopic discrimination is largely determined by diffusion into the plant including across the cell wall and then enzymatic fractionation by Rubisco. If the photosynthetic surface of the moss is covered with water, then access to carbon dioxide is limited by diffusion, and the photosynthetic enzyme Rubisco has less opportunity to discriminate against the isotopically heavier  $^{13}\text{CO}_2$  (Fig. 3b). This means that where moss is growing in predominantly wet hollows, or under thick montane clouds, the organic matter will be relatively enriched in  $^{13}\text{C}$  (Bramley-Alves *et al.*, 2015; Royles and Griffiths, 2015; Horwath *et al.*, 2019). Conversely, if the moss is growing in a drier and more exposed microclimate like a hummock, its photosynthetic surface will be exposed to air, greater discrimination will occur and cellulose will be more depleted in  $^{13}\text{C}$  (Bramley-Alves *et al.*, 2015; Royles and Griffiths, 2015). Furthermore, analysis of  $^{13}\text{C}$  and carbon's radioactive form  $^{14}\text{C}$ —to establish a date in which a sample was formed using accelerator mass spectrometry—can reveal how moss respond to climate variation overtime. For example,  $\delta^{13}\text{C}$  in moss shoots collected from the Windmill Islands, East Antarctica, were correlated negatively with wind speed and positively with air temperature (Clarke *et al.*, 2012). This dual isotope technique was then used to show that ozone depletion and climate change (Robinson and Erickson III, 2015) are drying these East Antarctic terrestrial communities with consequent changes in species favouring survival of cosmopolitan species like *Ceratodon purpureus* over the endemic moss, *Schistidium antarctici* (Robinson *et al.*, 2018). This technique allows scientists to monitor environmental changes in polar vegetation communities across space and time using carbon isotopic



signatures (Clarke *et al.*, 2012; Royles *et al.*, 2013a; Bramley-Alves *et al.*, 2015; Royles *et al.*, 2016; Robinson *et al.*, 2018).

Isotopic analysis can be used to determine plant communities at risk from (Robinson *et al.*, 2018), or favoured by (Royles *et al.*, 2016), climate change at broader scales or those impacted by buildings that can affect hydrology at local scales (Robinson *et al.*, 2018; Brooks *et al.*, 2019). Combining stable isotopic measurements ( $^{13}\text{C}$  and  $^{18}\text{O}$ ) can help to integrate photosynthesis, growth and water supply in polar moss species. However, more research, including growth under controlled environmental conditions (Bramley-Alves *et al.*, 2015) is needed to fully understand the oxygen and deuterium signals (Barbour, 2007; Royles and Griffiths, 2015).

Given that trees are absent from many high polar and alpine regions, this use of isotopes in mosses could provide proxy data to constrain climate models (Royles and Griffiths, 2015; Robinson *et al.*, 2018). An alpine example of such an application is seen in tropical montane cloud forest mosses and liverworts where isotopic composition of epiphytic bryophytes can reveal the position of the cloud immersion zone (Horwath *et al.*, 2019). The stable-isotope composition ( $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$ ) of these canopy-dwelling bryophytes reflects diffusive limitations due to surface water and, along with C/N content, provides a generic index for the extent of cloud immersion. From lowland to cloud forest,  $\delta^{13}\text{C}$  increased from  $-33\text{‰}$  to  $-27\text{‰}$ , while  $\delta^{18}\text{O}$  increased from  $16.3\text{‰}$  to  $18.0\text{‰}$ . Changes in stable isotopes thus have the potential to show where the cloud base is shifting upslope with climate change. Since changes in isotopes were apparent prior to diversity changes, they could be used to track epiphytic communities at risk, as well as to identify new areas suitable for growth. Combined radio- and stable-isotope analysis of bryophyte shoot cores could thus provide conservationists with a climatic proxy data to track changes in Antarctic terrestrial communities and potentially other bryophyte-dominated ecosystem change (e.g. Questions 12 and 41 in Cooke *et al.*, 2021; Table 2).

Unfortunately, not all moss species are suitable as climate proxies. Plant life form and anatomical differences can influence the extent to which water layers form and/or rates of diffusion of  $\text{CO}_2$  into leaflets of different moss species, and this will then be reflected in their effectiveness as climate proxies (Royles and Griffiths, 2015; Perera-Castro *et al.*, 2020). It should also be noted that mosses track climate in very localized microclimates and so multiple independent samples over a wide area are needed to infer macro climatic trends (Robinson *et al.*, 2018).

## Nitrogen isotopes and resource acquisition

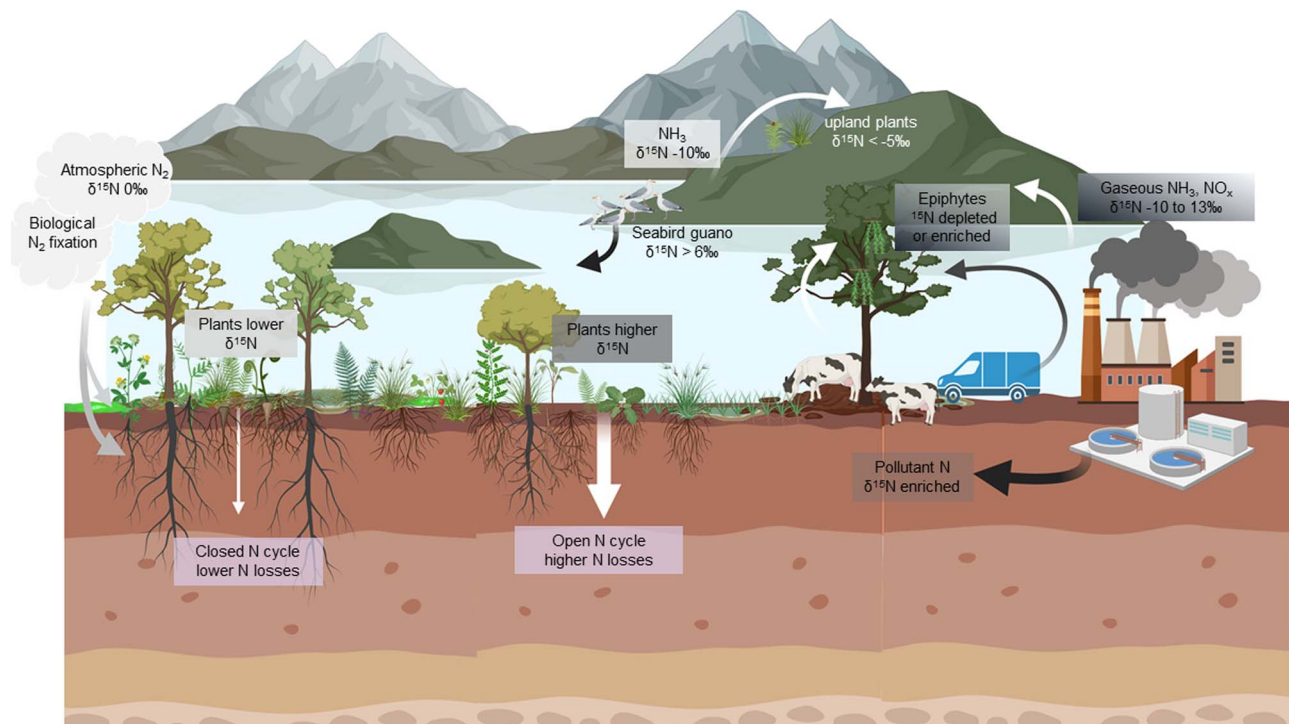
Plants growing in ecosystems that recycle proportionally more N in the vegetation-soil-vegetation loop are less enriched in  $^{15}\text{N}$  than in ecosystems with a more open N cycle

and loss of isotopically light N (Fig. 4). Across biomes, foliar  $\delta^{15}\text{N}$  signatures span mostly from  $-8\text{‰}$  to  $8\text{‰}$  from mesic to arid habitats, which broadly mirrors the increasing isotopic enrichment of whole-soil N (Handley *et al.*, 1999). Similarly, within a biome, a more open N cycle in the drier ecosystem (e.g. wetter vs. drier rainforests) results in isotopic enrichment of soil and plants (Handley *et al.*, 1999; Houlton *et al.*, 2006). Within an ecosystem, ecological guilds (early pioneer to late successional species) can have distinct foliar  $\delta^{15}\text{N}$  indicative of different N sources and N physiologies (Aidar *et al.*, 2003). In systems that have extreme isotope signals,  $\delta^{15}\text{N}$  can be used as a natural tracer. Seabird guano ( $\delta^{15}\text{N} \sim 10\text{‰}$ ) fractionates into isotopically depleted gaseous N and isotopically enriched liquid and solid N, allowing tracing of N sources with foliar  $\delta^{15}\text{N}$  ranging from  $-10\text{‰}$  to  $20\text{‰}$  in landscapes with large seabird rookeries (Erskine *et al.*, 1998; Schmidt *et al.*, 2004; Wasley *et al.*, 2012) (Fig. 4).

Stable-isotope ratios of N can be used for analysis of plant-plant interactions, for example the impact of invasive plant species on native species at the 'isoscape' landscape level. In a dune system, the  $\delta^{15}\text{N}$  signature of native species *Corema album* identified the influence of an invasive  $\text{N}_2$ -fixing *Acacia* species with isotopic enrichment in the native species together with a doubling in leaf N content (Hellmann *et al.*, 2016). Stable isotopes of N can also support interpreting stress physiology. For example, genotype comparisons of wild barley found that drought- and N-stress tolerant genotypes had the lowest  $\delta^{15}\text{N}$  values, reflecting the extent to which stress tolerant plants better retained N (Robinson *et al.*, 2000). Similarly, among taro (*Colocasia esculenta*) genotypes, the highest WUE correlated with the lowest  $\delta^{15}\text{N}$  (Gouveia *et al.*, 2019) (e.g. Questions 32 and 83 in Cooke *et al.*, 2021; Table 2).

## Short-term plant nitrogen uptake and $\delta^{15}\text{N}$

In the short term, N uptake via roots is most immediately reflected in xylem water. At wetter sites along a continental moisture gradient, foliar  $\delta^{15}\text{N}$  of  $\text{N}_2$ -fixing *Acacia* species overlapped with non-fixing *Eucalyptus* species, while *Acacia* xylem water was depleted in  $\delta^{15}\text{N}$  and distinct from  $\delta^{15}\text{N}$ -enriched xylem water of *Eucalyptus* (Soper *et al.*, 2015b). At drier sites, both tree genera had similar xylem  $\delta^{15}\text{N}$  signatures indicative of soil N use rather than biological fixation. Similarly, moribund vs. actively growing *Acacia* trees had isotopically enriched vs. depleted xylem water (Soper *et al.*, 2015b), and, by using xylem water (but not foliar  $\delta^{15}\text{N}$ ), biological  $\text{N}_2$ -fixation could be identified in *Prosopis glandulosa* in grassland-to-woodland transition (Soper *et al.*, 2015a). These patterns indicate that measurements of  $\delta^{15}\text{N}$  in xylem water can detect rapid shifts in plant N sources that, in turn, could preclude other measures of plant stress or changes in the availability of N in the ecosystem (e.g. Question 71 in Cooke *et al.*, 2021; Table 2).



**Figure 4:** Plant  $\delta^{15}\text{N}$  signatures integrate N relations. Lower  $\delta^{15}\text{N}$  signatures occur in mesic plant communities and systems with a more closed N cycle (less N loss). Higher  $\delta^{15}\text{N}$  signatures characterize systems with more open N cycle (greater N loss), such as arid environments and systems with high N input. Plant  $\delta^{15}\text{N}$  mirrors distinct N sources with isotopic signatures ranging from neutral (air  $\text{N}_2$ ), depletion or enrichment in natural ecosystems, and generated anthropogenically. Other effects on plant  $\delta^{15}\text{N}$ , such as environmental stresses or root specializations, are not shown.

### Longer-term responses—the nitrogen cycle and $\delta^{15}\text{N}$

Nitrogen is in the global spotlight because reactive N in the biosphere has more than doubled over the past century. Of the  $\sim 120$  Tg N that are annually fixed synthetically, half is lost from agricultural soils and, together with N derived from manures, other wastes, urban and industrial processes, impacts global ecosystems. Nitrogen deposition is considered the third most important driver of plant biodiversity loss in terrestrial ecosystems (Midolo *et al.*, 2019). Plant  $\delta^{15}\text{N}$  signatures can reflect the distinct isotopic signals of pollutant N sources in industrial, agricultural and urban environments. Tracking historic trends, aerial rainforest epiphytes showed a pronounced shift from minor  $\delta^{15}\text{N}$  depletion ( $-3\text{‰}$  to  $-1\text{‰}$ ) in herbarium specimens to strong depletion ( $-10.9\text{‰}$ ) in contemporary epiphytes exposed to air pollution from petrochemical and fertilizer industries, while  $\delta^{15}\text{N}$  of contemporary epiphytes at remote locations resembled herbarium specimens (Stewart *et al.*, 2002). Exposed to gaseous  $^{15}\text{N}$ -depleted  $\text{NH}_3$  and  $\text{NO}_x$  in agricultural landscapes with animal husbandry and crops, plant  $\delta^{15}\text{N}$  averaged  $-11.2\text{‰}$ , exposed to  $^{15}\text{N}$ -enriched  $\text{NO}_x$  of vehicle traffic, plant  $\delta^{15}\text{N}$  was  $13.3\text{‰}$ , while in natural ecosystems plant  $\delta^{15}\text{N}$  averaged  $5\text{‰}$  (Díaz-Álvarez *et al.*, 2018).

Thus, plant functional types can be explored for different investigations: mosses as ideal biomonitors for wet deposition, vascular epiphytes for dry deposition and terrestrial plants to monitor N saturation of soil. At the plant community level, a more closed N cycle with lower N turnover has less isotopic enrichment than an open N cycle, which can manifest in lower plant  $\delta^{15}\text{N}$  signatures, higher plant diversity and more efficient N use (Kleinebecker *et al.*, 2014), due to less isotopically enriched N sources and because plants discriminate against  $^{15}\text{N}$  in the presence of excess N (Marshall *et al.*, 2007). In oligotrophic alpine regions vulnerable to N enrichment, N and O isotopes in plant tissues could quantify atmospheric input of nitrate (Bourgeois *et al.*, 2019).

Nitrogen isotopes allow forensic investigation and mass balance calculations of N inputs at a landscape level. Plant  $\delta^{15}\text{N}$  mirrored the isotopic signal of municipal effluent in a production forest and wetland, which intercepted 65% of the applied N (Tozer *et al.*, 2005) while most of the remaining N (29%) entered a stream and could be tracked as isotopically distinct nitrate. Illustrating the detailed information derived from N isotope analysis,  $^{15}\text{N}$  enrichment of effluent-fertilized vegetation of up to  $20\text{‰}$  could be tracked with different stem parts that had distinct  $\delta^{15}\text{N}$  signatures, enabling temporal resolution of N sources (Tozer *et al.*, 2005). This is

of value to conservation managers as plant  $\delta^{15}\text{N}$  signatures integrate the isotopic composition of atmospheric, soil and water sources and document changing N relations over temporal and spatial gradients. Stable-isotope ratios of N can be a standalone tool or accompanied by other responses at ecosystem and organism levels, supporting observations of changing species composition, tissue N content, growth rate, phenology or mortality rate (e.g. Question 71 in *Cooke et al., 2021*; Table 2).

## Isotopic pulse labelling

A primary utility of stable isotopes is to identify plant resiliency (or susceptibility) to environmental stress caused by drought, heat waves, herbivory/disease, episodic disturbance or a combination of stress factors. Variation in the natural abundance of two isotopes of a given element can often uncover patterns of resilience when measured in plants and the surrounding environment. However, under many conditions, natural abundances do not contain enough variation to detect plant responses to environmental stress. Therefore, more sophisticated isotopic labelling approaches can yield critical information on plant resilience.

One of the most straightforward and effective labelling approaches in stable-isotope ecology is enriching irrigation water with a deuterium spike that can be traced in xylem water or leaf water. Previous deuterium 'pulse-labelling' experiments have uncovered contrasts in shallow water versus deep water exploitation among co-occurring dryland plant species (*Williams and Ehleringer, 2000*; *Schwinnig et al., 2002*; *West et al., 2007a*), among different periods of the growing season (*Williams and Ehleringer, 2000*), on different geomorphic surfaces (*Fravolini et al., 2005*), as well as different transit times of groundwater recharge and plant water uptake (*Evaristo et al., 2019*). Experiments using isotopically enriched water have also been used to instantaneously track the regeneration of leaf waxes (*Gao et al., 2012*) and water turnover rates in succulent-stemmed plants (*English et al., 2007*). Combined, these experiments provide a template of how plant conservation may be benefitted through isotopic labelling of water taken up by plants. For example, isotopic irrigation experiments could identify genotypes or species that are most resilient to changing seasonal precipitation patterns. Similarly, deuterium-labelling experiments can be used to detect whether certain plants can best regenerate and maintain leaf waxes that are critical for preventing passive water loss from leaves during heat waves, reduce intense UV exposure and minimize foliage herbivory (e.g. Question 22 in *Cooke et al., 2021*; Table 2).

Pulse-labelling experiments can also be used to track the fate of assimilated  $\text{CO}_2$  following photosynthesis by fumigating a plant, or part of the plant canopy with  $^{13}\text{C}$ -labelled  $\text{CO}_2$  (*Dawson et al., 2002*; *Staddon, 2004*; *Epron et al., 2012*). Pulse-labelling of  $\text{CO}_2$  can help quantify resource

partitioning under varying environmental conditions (*Joseph et al., 2020*) and help determine carbon allocation strategies and carbon transport to fungal mutualists (*Grimoldi et al., 2006*) that are often critical for plant fitness and conservation, which leads to an additional conservation question (Table 2): what are the best practices to monitor and manage plant-microbe symbioses in habitats impacted by disturbance and climate change? Stable isotopes can identify the presence of fungal mutualists following large-scale restoration projects following disturbance. For example, a pulse  $^{13}\text{C}$ -labelling experiment conducted in an old-growth Scots pine forest revealed that belowground carbon allocation to microbes is strongly modulated by soil moisture (*Joseph et al., 2020*). A recent study on Scots pine corroborates earlier studies that indicate that the transfer of recently acquired photosynthates to belowground sinks slows as soil-water content decreases (*Epron et al., 2012*). These results show the extent to which drought can disrupt plant-microbial mutualisms that may be important to successfully restore ecosystems impacted by disturbance or competition from invasive species (*Meinhardt and Gehring, 2012*; *Grove et al., 2017*). However, belowground allocation is also strongly governed by seasonality, temperature and plant phenology (*Epron et al., 2012*). For example, a pulse-labelling experiment revealed that belowground carbon allocation in 20-year-old beech peaked in midsummer but shifted to labile carbon storage under cooler temperatures at the end of summer (*Epron et al., 2011*). Thus, the seasonal timing in which  $^{13}\text{C}$ -labelling experiments are conducted is an important consideration (e.g. Additional Question 2, Table 2).

Ongoing advances in isotopic labelling approaches coupled with advances in genetic sequencing are providing new avenues for understanding basic plant biology in ways that can guide conservation. Among these are advances in the fields of metabolomics, transcriptomics and proteomics. Carbon isotope labelling experiments coupled with metabolomic, transcriptomic and proteomic profiling can be used to identify ways to improve nitrogen use in plants or uncover metabolic pathways for plant defence against microbes (*Pang et al., 2018*; *Zhang et al., 2018*) by characterizing metabolic fluxes, pathways and networks (*Chokkathukalam et al., 2014*; *Nakabayashi and Saito, 2020*). As with pulse chase experiments using  $\delta^2\text{H}$  of water or  $\delta^{13}\text{C}$  of air, compound-specific isotope labelling coupled with omics analysis can be challenging, labour intensive and costly to implement and analyse. However, rapid technical advances promise to provide new opportunities to develop conservation strategies of plants and ecosystems threatened with environmental changes.

## Future directions

Over the past several decades, stable-isotope ecology has evolved from the basic exploration of isotopic variation in nature to being recognized as a powerful tool for plant and



ecosystem responses to environmental change. Consequently, as conservation physiology continues to grow as an emerging discipline of plant ecology, stable-isotope techniques are increasingly gaining acceptance within the plant conservation physiology toolbox (Madliger *et al.*, 2018). Advances in mass spectrometry and tunable diode lasers (e.g. Schaeffer *et al.*, 2008) are not only increasing the spatial and temporal resolution of field-collected data, they are also leading to reduced costs associated with stable isotopic inquiry. In turn, these advances are leading to new avenues to couple stable-isotope analysis with other high-data capacity measurement tools including whole-genome sequencing (Chhetri *et al.*, 2019), remote sensing (Lavergne *et al.*, 2019) and phenology studies using automated camera sensors (Brown *et al.*, 2016). As conservation physiology evolves to include a broader spectrum of physiological tools and concepts (Cooke *et al.*, 2020), stable-isotope analysis has the potential to be on the forefront of conservation. To maximize this potential, we advocate for the construction of publicly accessible databases of isotopic abundance in plant tissues, soils and air (see Hayden *et al.*, 2014), continued standardization of laboratory protocols and standards and frequent horizon scanning to identify emerging challenges and opportunities for stable-isotope ecology and physiology to enhance conservation.

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## Supplementary material

Supplementary material is available at *Conservation Physiology* online.

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