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# Isotopic methods for non-destructive assessment of carbon dynamics in shrublands under long-term climate change manipulation

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**Running head:** Isotope techniques for ecosystem C science

***Tweet:*** *The pros and cons of carbon assessment methods using isotopes across climate change experiments in shrublands.*

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**Summary**

1. Long-term climate change experiments are extremely valuable for studying ecosystem responses to environmental change. Examination of the vegetation and the soil should be non-destructive to guarantee long-term research. In this paper, we review novel field methods using isotope techniques for assessing carbon dynamics in the plant-soil-air continuum, based on recent field experience and examples from a European climate change manipulation network.

2. Eight European semi-natural shrubland ecosystems were exposed to controlled warming and drought manipulations. One field site was additionally exposed to elevated atmospheric $CO_2$. We evaluate the isotope methods that were used across the network to evaluate carbon fluxes and ecosystem responses: 1) analysis of the naturally rare isotopes of carbon ($^{13}C$ and $^{14}C$) and nitrogen ($^{15}N$); 2) tracing changes in isotopic signatures in ecosystem compartments *in-situ*, by using pulse labelling with $^{13}CO_2$, soil injections of $^{13}C$- and $^{15}N$-enriched substrates, or continuos labelling with $^{13}C$-depleted $CO_2$ by Free Air Carbon dioxide Enrichment (FACE); and 3) manipulation and tracing the isotopic composition of soil substrates ($^{14}C$) in lab-based studies.

3. Questions related to long-term carbon turnover processes were investigated by natural $^{14}C$ signals, specifically $^{14}C$ signature of soil respiration gave insights into the decomposition of old soil carbon sources. Contrastingly, the stable isotopes $^{13}C$ and $^{15}N$ were used for shorter-term processes, as the residence time in a certain compartment of the stable isotope label signal is limited. $^{13}C$-labelling techniques exert a minimal physical disturbance, however, the dilution of the applied isotopic signal can be challenging, and the contamination of the field site with released excess $^{13}C$ can be a problem for subsequent natural abundance ($^{14}C$ and $^{13}C$) or label studies.

4. Based on the experience with the experimental work, we provide recommendations for the application of the reviewed methods to study carbon fluxes in the plant-soil-air continuum in long-term, large-scale climate change experiments.

**Key-words**: warming; drought; bomb-C; FACE; pulse-labelling; stable isotopes; $^{14}C$

# Inhalt



## Introduction

Global climate change scenarios predict that increased greenhouse gas (e.g. $CO_2$, $CH_4$ and $N_2O$) concentrations in the atmosphere will alter the periodicity and magnitude of drought events and will increase mean global temperatures by approximately 0.2 °C per decade (IPCC 2013). For the European continent this will manifest as drier summers in the South and increased precipitation in the North (IPCC 2013). Elucidating the consequences of such atmospheric changes for biogenic carbon fluxes is one of the main challenges for the scientific

community. Some models have predicted a positive feedback to climate change, resulting from higher increases in respiratory fluxes from ecosystems (e.g. carbon release through soil respiration) than in net primary productivity, which would lead to further increases in atmospheric $CO_2$ (Friedlingstein et al. 2006; Denman et al. 2007). To assess the likelihood of positive feedback, experimental studies that analyse the long-term adaptations of ecosystem carbon fluxes to climate change are critically needed. However, climate change experiments are often conducted at short or medium time scales due to funding constraints, or due to the limited life-span of the experimental plots, as repeated removal of samples often leads to disturbances and experimental artefacts in the studied system. Hence, there is a necessity for the maintenance of long-term experiments using non-destructive methods.

Carbon fluxes through the plant-soil-air continuum play a central role in soil carbon cycling. Drought and warming alter the quantity and composition of carbon inputs to the soil by changing plant available carbon and nitrogen sources (de Graaff et al. 2007; Leakey et al. 2009). Microbial growth in soil is generally constrained by available carbon, therefore qualitative and quantitative changes in rhizodeposition are likely to alter the activity of heterotrophic microorganisms and the rates of soil organic matter (SOM) mineralisation (Zak et al. 2000; Phillips et al. 2006). Consequently, aboveground to belowground fluxes might largely determine carbon emissions from ecosystems under the different climate change scenarios (Chapin et al. 2009).

Stable carbon isotope studies can give important insights into carbon fluxes through the plant-soil-air continuum with the minimal disturbance to the system. The isotopic carbon composition of compartments in this continuum is a result of the different isotope fractionation processes along the pathway from $CO_2$ fixation by plants to carbon allocation to soil (reviewed in Brüggemann et al. 2011). Thus, the analysis of the natural abundance of carbon isotopes in these compartments can give information about some processes related to photosynthesis and carbon losses through plant or soil respiration (Brüggemann et al. 2011). In addition, *in-situ* pulse labelling with the heavy stable carbon isotope ($^{13}C$) is a powerful tool to analyse short-term dynamics of carbon allocation to the soil with high resolution (Högberg et al. 2008; Epron et al. 2012; Reinsch & Ambus 2013). The recent development of techniques for $^{13}C$ analyses in specific compounds such as phospholipid fatty acids (PLFAs), amino-sugars, RNA and DNA, constitutes a remarkable advance in the studying of carbon cycling. These analyses allow for the examination of rhizodeposit utilisation by microbes or trophic interactions between functional groups (Ostle et al. 2003; Jin & Evans 2010; Ruess & Chamberlain 2010). The

application of these isotopic methods can therefore provide unique information about aboveground-belowground linkages and their alterations in response to climate changes.

In order to investigate long-term effects of climate change on shrubland ecosystems, an experimental network was established across Europe (the INCREASE network). Studying the response of shrublands to climate change is important, since they are representative ecosystems in Mediterranean and North European countries, where they play an important ecological role in preserving biodiversity (Verdú 2000; Wessel et al. 2004). In addition, land area covered by shrublands has dramatically decreased in temperate Europe during the past century, due to land use changes, increased pollution and eutrophication, and climate change (Fagúndez 2013). In Mediterranean regions, however, shrublands have increased their extension due to land abandonment (Fagúndez 2013).

Within the climate change network, common non-destructive methods were used across sites to ensure the comparison of treatment effects across different climatic regions (Beier et al. 2004; Mikkelsen et al. 2008). Evaluating the impact of climate change treatments on shrubland carbon dynamics was one of the main objectives of this experimental network, and thus a range of methodologies to quantify and trace distinct carbon pools and their fluxes have been applied since 1999. Priority was given to those techniques that minimise disturbances to vegetation and soil to guarantee long-term research.

Here, we review isotope methods that have been applied across this climate change experimental network to study ecosystem carbon dynamics in the plant-soil-air continuum. In particular, we focus on methodologies that: 1) analyse the abundance of naturally rare isotopes of carbon ($^{13}C$ and $^{14}C$) and nitrogen ($^{15}N$) in the different ecosystem compartments to evaluate their responses to climate change; and 2) trace experimentally-induced changes in the isotopic signatures to assess rhizodeposition utilisation by soil biota, by using either $^{13}CO_2$ pulse labelling, continuous labelling with $^{13}C$-depleted $CO_2$ from Free Air Carbon dioxide Enrichment (FACE), or injections of $^{13}C$- and $^{15}N$-enriched substrates in the field, and finally 3) manipulate and trace the isotopic composition of C-compounds to analyse C mineralisation by soil microorganisms in laboratory studies. Along-side the methods, data from the field studies are presented as accompanying illustrative boxes, and practical recommendations for the applications of these methodologies at large-scale climate change experiments are outlined in Table 1. The combination of the isotope methods with methods for *in-situ* quantification of

aboveground, root and fungal mycelia biomass will increase our understanding of climate change effects on carbon dynamics with the least possible disturbance.

**The experimental climate change network INCREASE**

The experimental network for the study of climate change impacts on European shrublands (INCREASE, 'An Integrated Network on Climate Research Activities on Shrubland Ecosystems') was established in 1998. The network is comprised of eight shrublands situated across a natural temperature gradient of mean annual temperature from c. 8 °C in the North to c. 16 °C in the South, and a rainfall gradient ranging from 510 mm to 1741 mm from East to West (see Fig. 1 in Reinsch et al. 2017). These sites represent Continental, Atlantic and Mediterranean shrublands. At each site, whole-ecosystem warming and drought treatments were applied in triplicates of 20 $m^2$ plots, by using automated retractable curtain constructions (see Beier et al. 2004 and Mikkelsen et al. 2008 for a full description). At one of the Danish sites (DK-BRA), a FACE treatment was installed, and combinations of the climate treatments were established and resulted in a plot size of 9 $m^2$ (Mikkelsen et al. 2008). Climatic conditions at the plot level (air temperature, humidity, soil temperature and moisture) were recorded in half-hour or hourly intervals, and main carbon pools and fluxes have been periodically monitored. Most frequent measurements include aboveground plant biomass and composition (Kröel-Dulay et al. 2015), litter production, soil respiration and net ecosystem carbon exchange (Beier et al. 2008; 2009; Lellei-Kovács et al. 2016). Measurements of litter decomposition, soil nitrogen mineralisation (Emmett et al. 2004) and soil microbial biomass and activity (Sowerby et al. 2005) have also been conducted with different periodicity.

## Methodologies using natural abundance of carbon isotopes

### 1 Ecosystem processes reflected by stable isotope fractionation ($^{13}C$ and $^{15}N$)

The relative abundance of the rare and heavy stable isotopes of nitrogen ($^{15}N$) and carbon ($^{13}C$) compared to the most abundant stable isotope, $^{14}N$ and $^{12}C$ respectively, is a signature that reflects the isotopic discrimination associated with gain and loss processes of a given entity. These signatures are expressed as the delta (δ) notation (e.g. $\delta^{13}C$ and $\delta^{15}N$ in ‰), which is the deviation of the rare isotope abundance in the sample compared to a reference material (Brand

& Coplen 2014; Muccio & Jackson 2009). The naturally occurring background level is termed 'natural abundance' of the given rare stable isotope (Berglund & Wieser 2011). Most natural processes (chemical, physical or enzymatically catalysed) discriminate against heavy isotopes (e.g. $^{13}C$, $^{15}N$, $^{18}O$), which in open systems results in an isotopically depleted product with comparably smaller concentration of the heavy isotopes than its corresponding substrate (Robinson 2001; Fry 2006). If the dominant process rate changes, or if the substrate is exhausted, then the δ value of the product (such as the plant leaf) may significantly change, due to the underlying fractionation. Delta notation is appropriate when dealing with natural samples that are not labelled with excess heavy isotopes (see next sections), and variations at the level of natural abundance are useful for evaluating natural discrimination processes. Importantly, natural abundance cannot be studied if 'overlain' by a study of labelling with heavy isotopes.

Decreases in soil water availability due to drought can alter the isotope signature of both carbon and nitrogen in the aboveground plant biomass. During drought stress, leaves reduce stomatal opening to preserve water. As this happens, the space that confines the air as an immediate source of $CO_2$ for photosynthesis (the sub-stomatal cavity) becomes a more closed system due to the restriction of the renewal of $CO_2$, and as a result a higher proportion of the heavy $^{13}C$ in $CO_2$ is fixed by Rubisco (C3 plants; Tcherkez et al. 2011). Hereby the discrimination against the heavy $^{13}C$ isotope is decreased. As a consequence, in plants with a C3 photosynthetic pathway a $^{13}C$ enrichment in the leaf occurs during drought stress (Cernusak et al. 2013). Indeed, the $^{13}C$ enrichment at the leaf level is related to an increased intrinsic water use efficiency (WUEi), the ratio of assimilation to stomatal conductance (Farquhar & Richards 1984; Donovan & Ehleringer 1994). Changes in soil water availability may also alter the leaf nitrogen isotope signature by changing the nitrogen availability with soil depth, and thereby the $^{15}N$ signature of the plant nitrogen source (Lloret et al. 2004). Since $\delta^{15}N$ is often analysed at the same solid sample as $\delta^{13}C$ by IRMS (Isotope Ratio Mass Spectrometer), interpretation of $\delta^{15}N$ can be a useful complement to understand the ecosystem processes. Nitrate and ammonium sources of different origin or at different soil depths can vary in $\delta^{15}N$ signature (Xue et al. 2009). Hence, if a drought event changes the vertical nitrogen availability in the soil, the plant nitrogen source can shift to a different soil depth possibly causing a change in $\delta^{15}N$ signature in the leaves. In general, an increase in $\delta^{15}N$ signature in the leaves indicates a progressive N saturation and/or N losses in the surrounding system because all major pathways of N loss (denitrification, ammonia volatilization and nitrate leaching) cause $\delta^{15}N$ enrichment of the remaining nitrogen (Peñuelas et al 2000). Interpretation of changes in leaf $\delta^{15}N$, however,

is not straightforward since leaf $\delta^{15}N$ signatures might largely depend on mycorrhizal associations and shifts in nitrogen sources between organic and inorganic compounds (the increase in plant δ15N values with aridity may also result from increasing reliance on recycled organic N sources as opposed to new inputs) under a drought or warming could influence the leaf $\delta^{15}N$ as well (Michelsen et al. 1998; Pardo et al. 2006; Andresen et al. 2009).

Across the field sites, the effects of warming and drought on plant $^{13}C$ and $^{15}N$ natural abundance was monitored over four years, starting two years after onset of the climate manipulations. Current year shoots or leaves were analysed for $\delta^{13}C$ and $\delta^{15}N$ immediately after each artificially prolonged drought. Plant material was dried at 70°C and ground to a fine powder before analysis for natural abundance values of $\delta^{13}C$ and $\delta^{15}N$ using isotope ratio mass spectrometry (IRMS). We expected to find higher $\delta^{13}C$ values: i) in drought treated plants (compared to control plots) and, ii) in plants growing at drier locations across the precipitation gradient (within a given plant species). Furthermore, we expected iii) the $\delta^{15}N$ to change in response to drought, as the nitrogen source (depth) is changed (at one location, within-species). Some significant effects of the drought treatment were observed on plant tissue $\delta^{13}C$ and $\delta^{15}N$ (Box 1). Differences between years (effect of time) were more pronounced than the effect of the drought treatment for *Populus alba* $\delta^{13}C$ (HU), *Erica multiflora* $\delta^{15}N$ (SP) and *Globularium alypum* $\delta^{15}N$ and $\delta^{13}C$ (SP). Only *Calluna vulgaris* showed a significant response to the drought treatment for $\delta^{13}C$ as hypothesized (Box 1A). For *C. vulgaris*, which was growing at several locations (UK-CL, NL and DK-MOLS), the $\delta^{13}C$ was higher at drier locations, when compared along the precipitation gradient, and also higher in the drought treatment, at the NL or UK-CL sites (Box 1B). Finally, we found no response of leaf $\delta^{15}N$ to drought or warming, however, *P. alba* had a much depleted $\delta^{15}N$ relative to the other species. We attribute these differences to species specific utilization of different nitrogen sources (perhaps more dependent on nitrate at the HU site), or different mycorrhizal associations with higher rates of isotopic fractionation (e.g. ericoid mycorrhiza in *E. multiflora* and *C. vulgaris*; Michelsen et al. 1998; Pardo et al. 2006).

## 2 Bomb-$^{14}C$ technique to asses sources of soil respiration

The analysis of natural $^{13}C$ abundance in $CO_2$ can be used to distinguish between autotrophic and heterotrophic components of soil respiration in some ecosystems where the sources of respired substrates (i.e. recent photosynthates vs. SOM) have contrasting $\delta^{13}C$ values. Changes

in $\delta^{13}C$ values can be observed after vegetation changes, e.g. where C4 plants are introduced in areas previously dominated by C3 vegetation, or vice versa, the so-called C3-C4 shift. In these situations, the $\delta^{13}C$ signature of plants and soil carbon may differ by up to 10 ‰ (Hanson et al. 2000). The partitioning of soil respiration between historical SOM and recent photosynthates can then be calculated using a linear isotope mixing models (Robinson & Scrimgeour 1995; Hanson et al. 2000). However, such vegetation shifts are rare in natural ecosystems, and therefore the use of $^{13}C$ natural abundance to differentiate between autotrophic and heterotrophic respiration is limited (but see Millard et al. 2008).

As an alternative to stable carbon isotopes ($^{12}C$, $^{13}C$), the natural radioactive $^{14}C$ abundance can be used to identify sources of soil respiration. Radiocarbon signatures of recent and older carbon are different as a result of the nuclear bomb tests in the atmosphere during the 1950/60s. These tests led to an increase in the $^{14}C$ content in the atmospheric $CO_2$ in the Northern hemisphere, which reached its maximum in 1963 ('bomb peak'). Ever since the subsequent atmospheric nuclear test moratorium, the 'bomb-$^{14}C$' content has decreased due to the dilution with fossil fuel-derived $CO_2$ in the atmosphere and its incorporation in ocean and terrestrial carbon pools (Trumbore 2009). Through that incorporation, the radiocarbon analysis of ecosystem fluxes provides information about the age of decomposed carbon substrates and can be used to differentiate carbon sources within ecosystems: recently plant-assimilated carbon (autotrophic component of soil respiration) should have a similar radiocarbon signature as the current atmosphere, while the radiocarbon content of older carbon released through SOM mineralisation (heterotrophic component) reflects the year of fixation of that carbon, again a mixing model solution. Several studies have successfully achieved the separation of sources of respiration across ecosystems using the 'bomb-$^{14}C$' method (Gaudinski et al. 2000; Cisneros-Dozal et al. 2006; Czimczik et al. 2006; Schuur & Trumbore 2006; Subke et al. 2011). In these studies, analysis of the $^{14}C$-$CO_2$ signatures of roots and SOM was performed under controlled conditions and collated with analyses of field gas efflux (the mixed pool). Radiocarbon analysis of soil or ecosystem respiration has been used to evaluate the response of a range of ecosystems to different factors of climate change, such as increasing temperatures, decreasing rainfall or permafrost thaw (Borken et al. 2006; Muhr & Borken 2009; Muhr et al. 2009; Schuur et al. 2009).

We tested the effect of experimental warming and drought on the natural abundance of $^{14}C$ in respired soil $CO_2$ at early stages of the climate manipulations at the Peaknaze field site (UK-PK). Our hypothesis was that drought increased heterotrophic respiration more than warming

in this seasonally waterlogged soil, due to a greater responsiveness of old soil carbon to drought relative to temperature as a driver (Bol et al. 2003; Domínguez et al. 2015). Therefore, we expected the greatest $^{14}C$-enrichment in the field-collected soil respiration samples from the drought plots. Soil efflux samples were collected in the late experimental drought period (September 2011), using a molecular sieve sampling system (Bol et al. 1995; Hardie et al. 2005; Hardie et al. 2009) attached to closed dark respiration chambers placed on the soil overnight. $CO_2$ was subsequently recovered from the molecular sieve traps for $^{14}C$ analysis by Accelerator Mass Spectrometry (AMS; Box 2). Soil and root samples were collected to conduct separate incubations to obtain the $^{14}C$-signatures of the heterotrophic and autotrophic respiration, respectively. These incubations were performed in leak-tight glass jars with a connection to the molecular sieve sampling system. The results revealed a high heterogeneity of the $^{14}C$ signature of the soil efflux with no significant effect of the warming treatment, and a trend towards the release of older carbon from the drought plots (although not statistically significant). By comparison with the known record of post-bomb atmospheric $^{14}C$-$CO_2$ concentration (Box 2), the carbon being released from the plots was estimated to have been fixed between six and eight years earlier (M. Dominguez, unpublished).

## Methods using *in-situ* $^{13}C$ labelling to study rhizodeposition utilisation

*In-situ* pulse labelling with the stable carbon isotope ($^{13}C$) is an efficient method for evaluating the time lag between carbon assimilation and $CO_2$ release from soil (Kuzyakov & Gavrichkova 2010). It can be applied to investigate a wide range of processes in the plant-soil-atmosphere continuum (Högberg et al. 2008; Brüggemann et al. 2011; Epron et al. 2012). With this method, a concentrated pulse of $^{13}C$-enriched or depleted substrate in the form of $CO_2$ or of a carbon-containing organic substrate, is released into the undisturbed ecosystem. Subsequently assimilation or heterotrophic consumption will transfer the labelled carbon, and the $^{13}C$ content of the product and organism will reflect the rate and the quantity of carbon transfer from one pool to another (Studer et al. 2014). Analysis of $^{13}C$ in specific compounds such as PLFAs is a specific tool to assess the utilisation of different carbon sources by different functional groups of soil biota. The development of $^{13}C$ labelling has increased the recognition of the central role that rhizodeposition plays in soil carbon cycling (Ostle et al. 2003; Jin & Evans 2010; Kuzyakov & Gavrichkova 2010; Dijkstra et al. 2013).

## 1 $^{13}C$-$CO_2$ pulse labelling

In $^{13}C$-$CO_2$ pulse labelling experiments, $^{13}C$ enriched $CO_2$ is released in closed, intact plant-soil systems during daylight hours, typically for 1.5 to 6 hours, where it is assimilated by the green plant biomass. Plant and soil samples are taken from unlabelled and labelled systems at different time intervals, with a higher sampling frequency within the first 48 hours after the labelling. The allocation of $^{13}C$ to belowground pools (roots, exudates, microbiota) is subsequently analysed, which allows the determination of the fraction of recently fixed carbon actively utilized by e.g. different microbial functional groups. Using $^{13}C$-$CO_2$ pulse labelling, several authors demonstrated that the flux of recently photosynthesized carbon to soil microbes occurs very fast, often within a few hours of $^{13}CO_2$ uptake (Rangel-Castro et al. 2005). The maximum incorporation of $^{13}C$ into microbial RNA occurs within four to eight days after the pulse (Ostle et al. 2003). Fungi typically show the greatest utilisation of plant-derived carbon within the first 48 hours after plant labelling. Lower $^{13}C$ enrichment in bacterial biomarker PLFAs indicates a delay in the utilisation of plant-derived carbon by bacteria, or a greater dependence of bacteria on carbon sources different from recently-fixed carbon. Gram positive bacteria and, in particular actinomycetes, are known to rely less on plant-derived carbon than Gram negative bacteria (Butler et al. 2004; Treonis et al. 2004; Balasooriya et al. 2008; 2013; 2014). The levels of allocation of belowground fixed carbon and the subsequent utilisation by microbes might be affected by a range of factors such as the seasonality of plant activity. Usually, more carbon is allocated belowground towards the end of the growing season (Högberg et al. 2010; Balasooriya et al. 2013), under exposure to elevated atmospheric $CO_2$ concentrations (Denef et al. 2007; Jin & Evans 2010; Reinsch et al. 2013), under drought conditions (Fuchslueger et al. 2014) or in plants grown on fertile soils (Denef et al. 2009; Paterson et al. 2011).

In the climate change network, several pulse-labelling experiments have been conducted in combination with $^{13}C$-PLFA analyses to study rhizodeposit utilisation by microbes. At the Clocaenog site (UK-CL) we aimed to study the utilisation of rhizodeposits along a soil moisture gradient, by applying a $^{13}C$-$CO_2$ pulse during the late growing-season (August 2011). We used transparent domes of 50 cm diameter and 100 cm height, enclosing individual *C. vulgaris* plants. We applied repeated pulses of $^{13}C$-$CO_2$ (99 atom% $^{13}C$ = 99% $^{13}C$ + 1% $^{12}C$) over eight hours (Box 3). The domes were sealed to a frame which was inserted into the ground at least ten days before the pulse, and had several sealed septa to collect gas samples to estimate the concentration of the $^{13}C$-labelled $CO_2$. Plant leaves and soil from the rooting zone were

collected at different times after the labelling, using a higher sampling frequency during the first hours after the pulse. Soils were freeze-dried, sieved to ≤ 5 mm and PLFAs were extracted. Fatty acid methyl esters (FAMEs) were analysed by gas chromatography combustion-isotope ratio mass spectrometry (GC-c-IRMS). The main challenge was the low recovery of $^{13}C$ label in the belowground compartment, especially in individual FAMEs. Despite the applied $^{13}C$ concentration of 99 atom%, the apparent low photosynthetic rates combined with the excessive dilution of the $^{13}C$ label in the large carbon pools of unlabelled woody branches and root- and microbial biomass resulted in an overall low level of $^{13}C$ enrichment in the FAMEs (Box 3). Similar patterns have also been observed in other pulse labelling experiments (Griffith et al. 2004).

Three pulse-labelling events were conducted at the Brandbjerg site (DK-BRA,) between 2010 and 2013 (Box 3). The Brandbjerg experiment consists of drought and warming manipulations in combination with ambient and elevated levels of $CO_2$ concentration. The developed experimental setup for pulse-labelling aimed i) to be easily deployable in remote areas, ii) to distribute labelled $^{13}C$-$CO_2$ to as many plots at the same time as possible to ensure similar and constant conditions for $CO_2$ uptake by the vegetation, and iii) to ensure constant $CO_2$ concentration available to the vegetation throughout the labelling period. Therefore, a mobile flow-through system suitable for continuous $^{13}C$-$CO_2$ delivery was developed (Box 3): A gas-tight vinyl balloon (~3 m diameter) was filled with $CO_2$ free synthetic air and mixed with $^{13}C$-$CO_2$ (50 or 99 atom%) that supplied the transparent chambers enclosing the vegetation of interest with air over the duration of the experiments ranging from 4 to 7.5 hours. Air was pumped continuously through gas tight tubing via electric diaphragm pumps (Reinsch & Ambus 2013). The first experiment was conducted at the end of the growing season (October 2010), when we observed the highest allocation of carbon belowground as measured by $^{13}C$ in soil respiration (Reinsch et al. 2014). The second experiment was conducted in the spring (May 2011) and showed a major allocation of carbon to aboveground structures under elevated atmospheric $CO_2$ concentration, but carbon allocation to belowground structures was higher in drought plots than in untreated control plots. The allocation of recently-assimilated carbon under warming conditions was similar to that under ambient conditions. The last experiment, conducted in early season 2013 (June), was performed during a period with impeded photosynthetic activity and indicated that labelling performance is poor when vegetation is recovering from harsh winter conditions with bare frost or severe drought conditions (Box 3).

Thus, it is important that the vegetation of interest displays green, photosynthetically active structures to facilitate $CO_2$ uptake and sufficient labelling of ecosystem carbon pools.

Our studies illustrate the complexity of controlling *in-situ* pulse-labelling experiments in ecosystems dominated by woody plants, which is even more challenging with $^{13}C$-$CO_2$ than with $^{14}C$-$CO_2$ because of their respective atmospheric backgrounds and detection limits (Epron et al. 2012). Ideally, $^{13}C$ doses for *in-situ* use should be carefully tested in trials, considering the nature of the studied vegetation and the compounds to be analysed. If e.g. specific compounds of the soil microbial biomass are the main interest, then strong isotopic doses should be applied, and it is advisable to deploy the $^{13}C$ pulse when plants naturally allocate carbon belowground e.g. when preparing for winter. The $^{13}C$ signal can be increased by using highly labelled $^{13}C$-$CO_2$ (99 atom %). However, the usage of a highly enriched $CO_2$ can potentially lead to blurry signals and has to be applied with caution (Watzinger 2015). Furthermore, $^{13}C$-$CO_2$ concentration inside the labelling chamber should be as close as possible to ambient values, because unrealistic high $CO_2$ concentration will change plant $CO_2$ uptake. Repeated moderated $^{13}C$-$CO_2$ applications during longer exposure times might be more appropriate, but inside closed transparent chambers, temperature and humidity may increase if the labelling period is prolonged, which also affects photosynthetic processes (Epron et al. 2012). Losses of $^{13}C$ due to physical diffusion and adsorption/desorption into the chamber and tubing material should also be considered. In particular, the back-diffusion of the $^{13}CO_2$ from the soil to the atmosphere which entered the soil pores during the labelling might confound the interpretation of measured belowground respiration (Subke et al. 2009; Selsted et al. 2011). However, when applied properly, the insights into terrestrial carbon allocation will be detailed and novel (Box 3).

## 2 Free Air Carbon dioxide Enrichment (FACE)-labelling

An alternative method for $^{13}C$ pulse-labelling of vegetation and whole-ecosystems is to utilize the $^{13}C$-depleted $CO_2$ in already planned or ongoing FACE experiments. The FACE technique has through decades been used within cropping systems (Kimball 2016), grasslands (Hovenden et al. 2014; Reich et al. 2014; Mueller et al. 2016) and forests (Terrer et al. 2016) experiments, with the primary goal of assessing potential carbon dynamics and enhancement of plant growth (Andresen et al. 2016). As a side effect, the change in carbon isotopic composition of vegetation exposed to FACE-treatment can be used to trace freshly assimilated carbon into soil microbial

biomass, fauna and organic carbon pools. This approach was used at the Brandbjerg site (DK-BRA). The $CO_2$ used to elevate concentrations of atmospheric $CO_2$ to 510 ppm had $\delta^{13}C$ values ranging from –3.0 to –36.7 ‰ throughout 8 years of experimental treatment, with and overall mean of -26.1 ‰. The source of the $CO_2$ supplied by Air Liquide (Air Liquide, Denmark) was most often a brewery surplus $CO_2$ as a chemically obtained side product. The FACE mixing of the added $CO_2$ with ambient $CO_2$ in the moving air mass resulted in a $^{13}C$ depletion ranging from -6.7 to -15.6 ‰. On average, this equals a depletion of $CO_2$ in FACE plots of -4.8 ‰ relative to the atmospheric -8 ‰ average. Ecosystem carbon pools became depleted accordingly, and the FACE $^{13}C$ depletion acted as a long-term persistent isotope labelling. As a result, soil fauna (Enchytraeids) sampled from each of the climate-treated plots was significantly depleted in $\delta^{13}C$ by -0.5 to -2.0 ‰ in the $CO_2$ treatments (Andresen et al. 2011). This was due to translocated $^{13}C$ substrate through the food web, starting with plant assimilation of $^{13}C$-depleted $CO_2$, followed by plant root exudation and microbial utilization of the $^{13}C$ depleted substrate and eventual digestion of microbes by enchytraeids. Hereby the freshly supplied carbon source was recognized to be transferred in the natural setting, within a given time scale. Also microbial biomass and PLFAs had different baseline of $^{13}C$ content in ambient (not-treated) plots compared to $CO_2$ treated plots (Andresen et al. 2014). This was used for the calculation of $^{13}C$ enrichments for each PLFA biomarker individually, also illustrating the pathway of newly-assimilated carbon into microbial biomass. A drawback of the $^{13}C$-FACE label is again contamination of the surroundings, as even short and small un-planned draft winds can carry the depleted label onto ‘ambient’ plots, and these will most likely be ‘contaminated’ with $^{13}C$ (though not markedly exposed to high $CO_2$ concentrations) after some years of FACE activity. Therefore, one needs to collect reference material for the ‘natural abundance’ level well away from the FACE experiment.

## 3 In situ injection of $^{13}C$-enriched substrate solutions

As a much more localized approach to a specific area, *in-situ* addition of $^{13}C$- and $^{15}N$-enriched substrates directly below the soil surface can be used to assess the competition for the substrate between i) plants and soil microbes, ii) microbial groups, and iii) the effects of the climate change treatments upon the competition for carbon or nitrogen substrates. Much research has focused on the sharing of nitrogen sources between plant and microbes (Kuzyakov & Xu 2013) using *in-situ* soil injections of $^{15}N$ labelled inorganic nitrogen (ammonium and nitrate) or

organic nitrogen (amino acids) (Bardgett et al., 2003; Sorensen et al., 2008). Once amino acids with dual labelled compounds ($^{15}N$ and $^{13}C$) were available for experimental use, double-labelled substrate was used to explore e.g. plant uptake of intact amino acids (Näsholm et al. 2009; Rasmussen et al. 2010), and microbial utilization of carbon substrates (Dungait et al. 2013; Rinnan & Baath 2009).

In a labelling experiment at the DK-BRA site, amino acid injections into the soil were conducted to analyse the impact of the climate treatments on the uptake of free amino acid nitrogen by plants and soil microbes. Dual-labelled glycine ($^{13}C_2{}^{15}N$-glycine: 99 atom% $^{13}C$ - of both carbon atoms - and 99 atom% $^{15}N$) was added to 20 × 20 $cm^2$ sub-plots (Andresen et al. 2009). Each sub-plot received 0.1 L of re-demineralised water labelled with 0.027 g glycine, corresponding to 687 mg glycine $m^{-2}$ (223 mg C $m^{-2}$ or 0.016 mg glycine $g^{-1}$ dry weight soil). The label was injected into the soil just below the soil surface with a syringe moved among 16 evenly spaced points of a template, placed on top of the vegetation (Andresen et al. 2009). One day (c. 24 h) after labelling with glycine, soil cores were sampled from the soil surface to 15 cm depth, for determining the relative uptake of the amino acid in plant roots (IRMS solid sample) and soil microbes. As in many other soil labelling experiments, the largest label recovery (measured by $^{15}N$ recovery since respiratory losses of $^{13}C$ remain unknown) was found in the total microbial biomass compared to total plant biomass (Kuzyakov & Xu 2013). A subsample of fresh soil was extracted with re-demineralised water, and another set of subsamples was first vacuum-incubated with chloroform for 24 hours to release microbial carbon and nitrogen (Joergensen & Mueller 1996; Brookes et al. 1985), before extraction with re-demineralised water. A third subsample of soil was freeze dried (lyophilized) and later used for PLFA extractions. The $^{13}C$ enrichment in marker PLFAs thus indicated the activity (vitality) of the specific microbial group (Watzinger 2015). We found that bacteria opportunistically utilised the freshly added glycine substrate, i.e. incorporated $^{13}C$, whereas fungi showed only minor or no glycine derived $^{13}C$-enrichment (Andresen et al. 2014). In comparison, $^{13}C$ traced into the microbial community via the $^{13}C$-$CO_2$ pulse label at the same site (DK-BRA) also reached the bacterial community first. Bacteria showed high $^{13}C$ enrichment compared to fungal groups (Reinsch et al. 2014). This suggests that *in-situ* injection of $^{13}C$ substrates might be a plausible alternative to mimic rhizodeposition effects. With the direct addition of $^{13}C$ label to the soil a strong labelling of the microbial community was more easily achieved than with the indirect $^{13}C$ labelling of microbes via plant assimilated $^{13}C$-$CO_2$ (Box 3).

## Use of labelled carbon-compounds to analyse carbon mineralisation by soil microorganisms

Since soil microorganisms have an important role in controlling the availability of nutrients via mineralisation of SOM, our understanding of how microbial functioning in the ecosystem is altered by global change must be improved (Grayston et al. 1997). Microbial catabolic diversity of a soil is directly related to the carbon decomposition function within a soil and potentially provides a sensitive and ecologically relevant measure of the microbial community structure (Garland & Mills 1991). Subsequently, multiple assays have been developed to generate community level physiological profiles (CLPP) that can act as fingerprints of microbial function. Three approaches for measuring CLPP in soils are reported in the literature: (i) Biolog (Garland & Mills 1991); (ii) a substrate-induced respiration (SIR) technique (Degens & Harris 1997); and (iii) MicroResp (Campbell et al. 2003). These methods are all based on quantifying $CO_2$ respired during the mineralisation of organic carbon compounds that vary in size, charge and structural complexity. The first approach, Biolog MicroPlateTM (Biolog), assesses the catabolic diversity of soil organisms using a microtitre plate by incubating a soil culture in the presence of nutrients and 95 different carbon substrates; respired $CO_2$ is used to reduce a tetrazolium violet salt, which results in a colour change that can be quantified colorimetrically (Garland & Mills 1991). This approach, however, has been criticized for bias towards fast growing organisms that thrive in culture (Preston-Mafham et al. 2002). In response to the criticism of the Biolog method, Degens & Harris (1997) developed a method based on SIR where individual substrates are added to intact soil and evolved $CO_2$ is sampled and quantified. Finally, Campbell et al. (2003) combined aspects of both methods (MicroRespTM) where the response to carbon substrate addition to soil is measured colorimetrically using a cresol red indicator dye in a microtitre plate format.

Community level physiological profiling of soils samples collected from all treatments across the network was conducted to determine the catabolic utilisation profile, turnover and pool allocation of low molecular weight (LMW) carbon compounds by using a selection of $^{14}C$-labelled substrates. This method enabled the attribution of respired $CO_2$ to specific metabolic processes that facilitates the quantification and qualification of microbial mineralisation kinetics of substrates varying in structural complexity and recalcitrance. The kinetics of microbial $^{14}C$-$CO_2$ evolution can be described using a first order exponential decay model (Box 4). The number of terms used in the exponential decay model can be used to explain how microbial kinetics relates time, substrate complexity and carbon pool allocation to, for example,

rapidly cycled labile soil solution carbon, microbial structural carbon and recalcitrant extracellular soil organic carbon (Kuzyakov & Demin 1998; Nguyen & Guckert 2001; Boddy et al. 2007). Attribution of modelled carbon pool sizes and turnover rates to biological function are not only time and substrate dependent. Therefore, soil physical, biological and chemical interactions may be miss attributed to biological function. Indeed, the lack of knowledge and techniques available to examine the interaction between discrete carbon pools (Glanville et al, 2016). Using the half-life of $^{14}C$ labelled carbon in soil solution we were able to examine the environmental gradient of the warming treatment across the climate change network and identified that temperature becomes rate limiting for microbial uptake of carbon from the soil solution pool at < 10.5 °C. We also showed that experimentally manipulated warming simply speeds up the catabolic utilisation of labile LMW carbon in a predictable pattern (Box 4).

## Conclusions and recommendations

Stable isotope studies provide insightful information about carbon (and nitrogen) fluxes through the plant-soil-atmosphere continuum with minimal disturbance to the system, and contribute to advance our understanding of climate change impacts on aboveground-belowground linkages. However, their application is not exempt from difficulties and disadvantages. To keep a high caution and avoid mistakes, our collective recommendations for applying the described methods are provided and addressed in Table 1.

*In-situ* pulse-labelling studies are powerful to analyse short-term carbon fluxes in the plant-soil system, but there are major seasonality constraints to the distribution of the label throughout the ecosystem compartments, *i.e.* the seasonality of carbon allocation belowground due to changing plant activity, or the plant health status which determines the amount of tracer entering the system. A significant challenge was the achievement of sufficient $^{13}C$ enrichment in microbial biomass in *in-situ* $^{13}C$-$CO_2$ pulse-labelling studies, where the pools of background carbon in the studied compartments were high and hence diluted the $^{13}C$ signal. This was less of a problem for $^{14}C$ studies, due to more sensitive analysis methods.

Importantly, field plots previously ‘contaminated’ by highly enriched isotope labelling should be considered potentially inoperable for further scientific isotope studies using the natural abundance approach. However, plant and soil structures remain largely undisturbed. In outlook for setting up a large-scale climate manipulation, areas that have not been previously used for experimental work with isotopes should consequently be selected. In particular, the ‘bomb-C’

553 method is very sensitive to the contamination of soil or plant samples with $^{14}C$-enriched
554 material, and thus its application should be limited to sites and facilities where no $^{14}C$-labelling
555 work has been conducted. Additionally, it should be noted that any history of fertilization might
556 also alter the natural isotope abundance of ecosystem compartments (in particular $^{15}N$
557 signatures), potentially confounding experimental results. Furthermore, military training
558 grounds, public recreational activity and vicinity of traffic are known to potentially contaminate
559 the soil with 'artificial compounds' which might interfere with delicate measurements on HPLC
560 and GC-MS systems.

561 Incubation studies with isotope labelled carbon compounds *in-situ* or *in-vitro* are relatively fast
562 to conduct and produce insightful data. The rapid utilisation of labelled substrates by soil
563 microorganisms, occurring immediately upon application, is a controlling factor for the timing
564 of the experimental work both at field and lab conditions. Hence, a sampling scheme needs to
565 be carefully planned before experiment initiation. Furthermore, pre-obtained knowledge of site
566 specific plant and microbial activity, substrate affinity and natural substrate concentrations is
567 important for planning any tracer application experiment (Table 1).
568

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570

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899

900 ## Table

901 **Table 1.** Suggestions and advice to consider when applying isotopic methods for the study of
902 carbon fluxes in the plant-soil system.

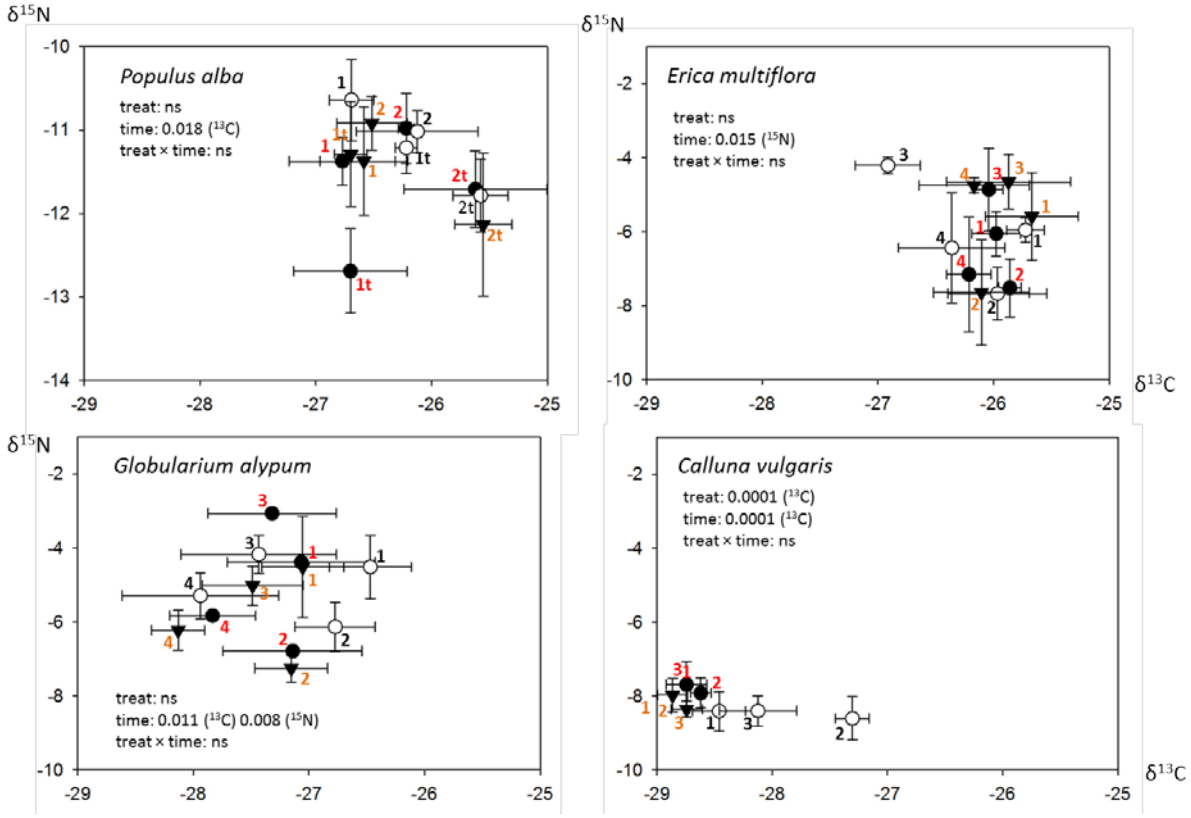
| Method | Expenses (cost) | Advice (do's and don'ts) | Before you start | Data analysis hint | Time spent |
|---|---|---|---|---|---|
| **Bomb-C (natural $^{14}C$ abundance)** | High (AMS analysis); Equipment for $CO_2$ sampling is relatively cheap (closed chambers, carbon free pump, batteries, and molecular sieve system). An IRGA is also required. | - Avoid materials and labs with possible $^{14}C$ contamination<br>- If soil $CO_2$ is to be analysed in the field, long incubation times are required to get sufficient $CO_2$ for AMS analysis (typically >1 ml).<br>- Think carefully about the soil depths to be analysed, and take the sample consistently. $^{14}C$ signatures might vary strongly along few cm in the soil.<br>- If bulk soil $^{14}C$ is to be analysed, try to remove the roots as much as possible, because of their contrasted $^{14}C$ signature. | - Discuss your experimental setup and objectives with the Radiocarbon facility staff.<br>- If you are not sure about potential $^{14}C$ contamination in your lab, use another lab or make a swipe test.<br>- Plan carefully the minimum number of samples required, as AMS analysis are expensive.<br>- Make previous trials to assess the incubation times required to get a sufficient $CO_2$ sample<br>- Go through the whole process of sample preparation with a trial sample. | - Discuss your results with the Radiocarbon facility staff. | - Processing time depends on the type of sample, although is usually low; determination by AMS may take several months depending on the facility. |
| **In situ $^{13}C$ pulse-labelling** | $^{13}C$- enriched compounds used for labelling and as standards are usually expensive; $^{13}C$ determination by IRMS is much cheaper than $^{14}C$ by AMS, although more expensive than $^{14}C$ by liquid scintillation ($^{14}C$ labelling). | - Consider the target pools to be analysed and plan your doses consequently. Take the potential dilution of the label by the unlabelled root system or soil carbon pool into account.<br>- Think about the trade-off between faster and stronger or weaker and longer $^{13}C$ pulses.<br>- If your study requires a high $^{13}C$ enrichment, mind the potential risk of contaminating the site.<br>- Avoid above ambient $CO_2$ concentrations in the chamber.<br>- If you need to monitor $CO_2$ during your pulse, remember that IRGAs are rather insensitive to $^{13}CO_2$.<br>- High sampling frequency immediately after the pulse application is recommended. | - Test your chamber and tubing materials for adsorption / desorption effects, and ensure these are without carbon content (use PTFE (Teflon) tape, not gluing paper-based).<br>- Make a previous trial if possible and go through the whole process of sample preparation. | Report the label addition per area: g $^{13}C$ $m^{-2}$. | - Pulse labelling experiments are usually short, but intensive. High sampling frequency after the pulse is very time-consuming.<br>- Sample processing depends on the type of sample and number of replicates.<br>- Experiments requiring root washing or microbial compound extraction are time consuming. |
| **Natural abundance of isotopes ($^{13}C$ and $^{15}N$)** | Rather cheap IRMS analysis. | - Make sure the history of sampling site is known (previous labelling experiments?) | - Avoid sample contamination.<br>- Be aware that FACE can dilute the isotopic signal, most $CO_2$ enriched systems use $^{13}C$ depleted sources, | - Make sure the experiment is feasible with sufficient $\delta^{13}C$ shift and fractionation expected to be strong enough to | - Sampling time and grinding / weighing of sample.<br>- Analysis usually done at dedicated |

| | | | | | |
|---|---|---|---|---|---|
| | | | because this is cheaper. | measure, base this on known discrimination at certain points in the carbon and nutrient cycle. | natural abundance facility. |
| **$^{14}$C-substrates** | Analysis of the trapped $^{14}C$-$CO_2$ is relatively cheap. | - High risk of contaminating lab equipment. | - You need to work in a dedicated $^{14}$C lab safely away from the natural abundance facility. | | - Continue sampling until decline in emission is level, this ensures better model fit. |
| **$^{13}$C-injection *in situ*** | IRMS of dry matter plant material and soil cheap.<br>The GC-c-MS of PLFAs for determination of $^{13}$C-enrichment of individual PLFAs requires a specialised lab. | - Contamination risk of $^{13}$C leaching is present, but smaller to our judgement than from $^{13}C$-$CO_2$ experiments.<br>- Do not use areas dedicated to natural abundance work. | - Labelling intended for soil microbial components is more intense from $^{13}$C liquid substrate *in-situ* injection than from $^{13}C$-$CO_2$ pulse labelling. | | - Soil sampling is destructive, consider to have several parallel plots to harvest an undisturbed plot at each sampling event.<br>- Sample handling from field work until the extraction takes a few days so plan only one sampling event per week if possible. |

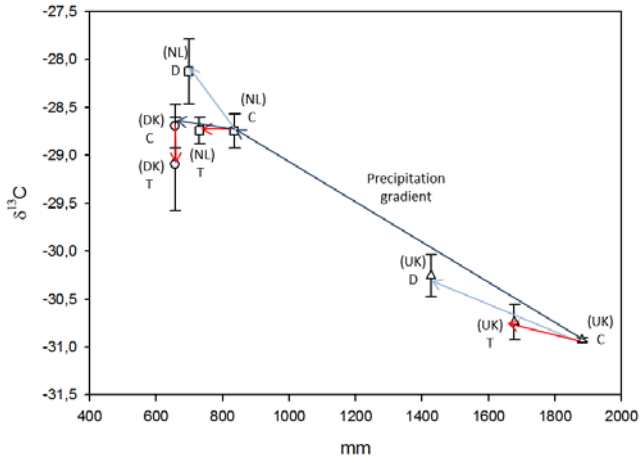
903

## Box 1. Isotopic signal of plant leaf responses to precipitation

**A**



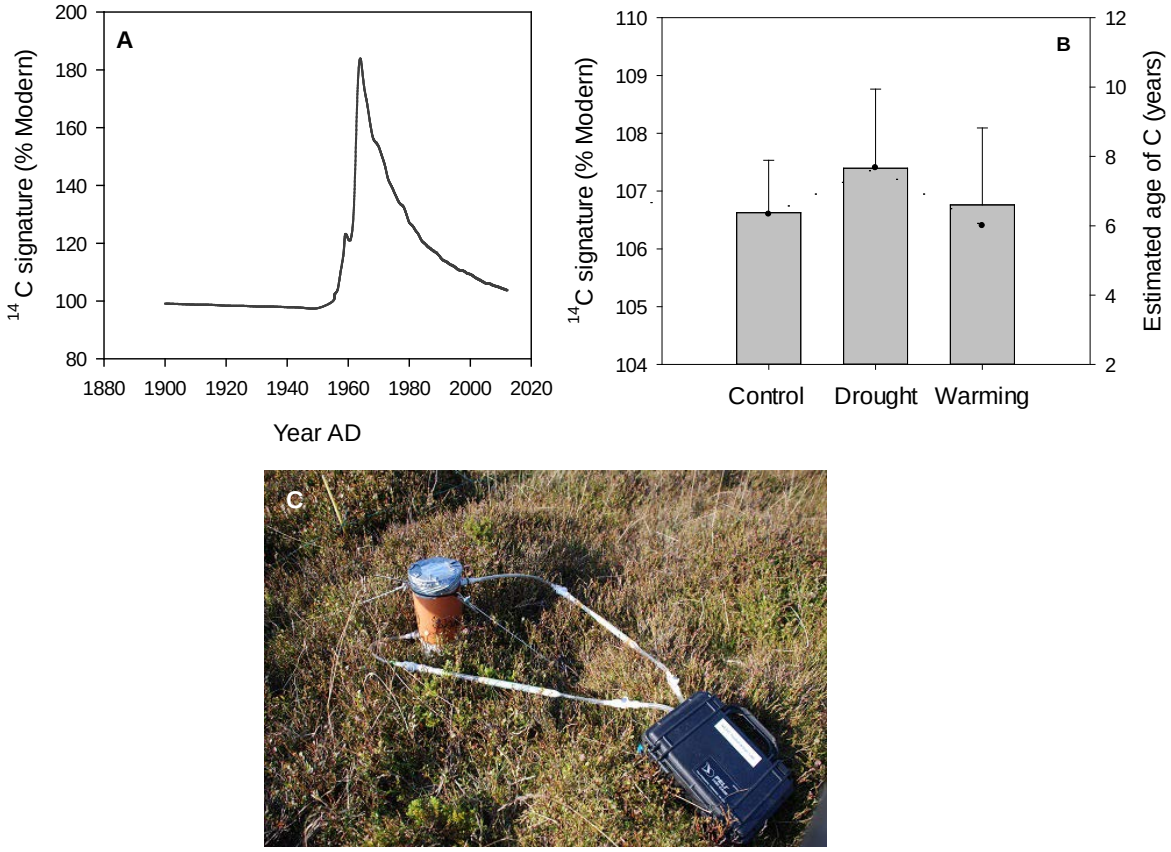

**B**




Stable isotopes in aboveground plant material: $\delta^{13}C$ and $\delta^{15}N$ from isotopic ratio mass spectrometry (IRMS) analysis of solid samples. **A:** Leaves and twigs (t) from *P. alba* (HU), *E. multiflora L.* (SP), *G. alypum L.* (SP) and *C. vulgaris* (NL); filled circle ● is control, open circle ○ is drought treatment, ▼ is warming treatment. P-values indicate effects of treatment, year, and the interaction of these factors on $^{13}C$ or $^{15}N$, analysed by two-way ANOVA; ns is non-

914 significant effect. Number indicates year (2001=1, 2002=2, 2003=3 or 2004=4). Species (site)
915 differences and annual differences are stronger than treatment effects. **B:** $\delta^{13}C$ of *C. vulgaris*
916 versus annual precipitation of the previous year. Within each site, leaf $\delta^{13}C$ was higher in the
917 drought treatment in comparison to control. Across sites, plants from drier sites (lower
918 precipitation) show higher leaf $\delta^{13}C$ values. The response to the drought treatment is the same
919 as moving to a drier site.
920

## Box 2. Impact of warming and drought on the $^{14}C$ signature of soil respiration

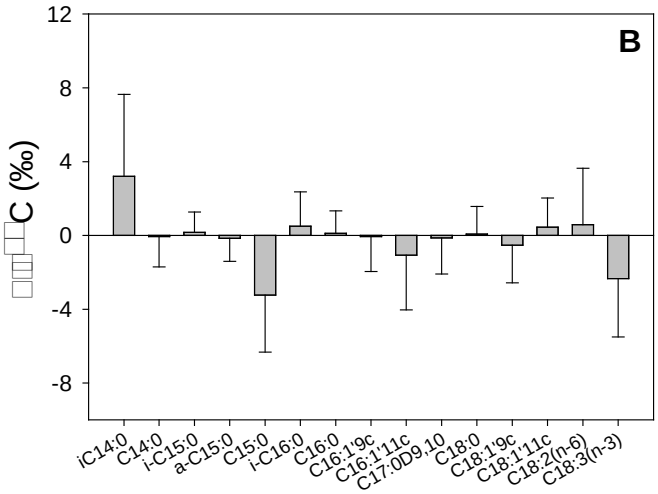


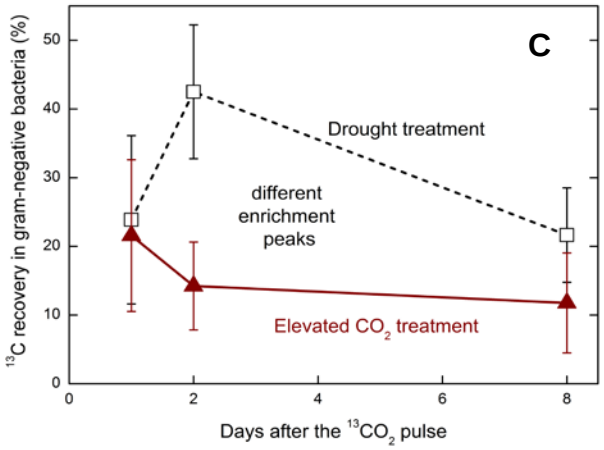

Closed dark respiration chambers and a molecular sieve sampling system was used in the field (UK-PK) to collect $CO_2$ of the soil efflux for the analysis of its $^{14}C$ signature by Accelerator Mass Spectrometry (AMS). **A:** Recent and older carbon sources have contrasting radiocarbon ($^{14}C$) signatures as a result of the nuclear bomb tests in the atmosphere during the 1950/60s. These tests led to a global increase in the $^{14}C$ content in the atmospheric $CO_2$, which reached its maximum in ~AD1963. The unit for $^{14}C$ signature (% Modern) is a measurement of the deviation of the $^{14}C/^{12}C$ ratio of a sample from the "Modern" standard, which is defined as 95% of the radiocarbon concentration (in AD 1950) of a reference material (NBS Oxalic Acid I, SRM 4990B), adjusted to a $\delta^{13}C$ reference value of –19 ‰. **B:** The $^{14}C$ signature of the soil efflux measured at the site (bars, left axis) was highly heterogeneous (ranging from 105.49 to 110.13 % Modern; values of > 100 % Modern suggest that a substantial component (and potentially all) of the carbon was trapped by photosynthesis during the post-bomb era i.e. since ~AD 1955), with no significant effect of the warming treatment, and a trend towards the release

938 of older carbon in the drought plots. On average, the carbon being released from the plots had
939 been fixed from the atmosphere between six and eight years earlier (line, right axis). Incubations
940 confirmed that the carbon respired by roots (mostly of *V. myrtillus L.*) was recently fixed
941 (similar $^{14}C$ signature as the atmosphere at the time of sampling), while the carbon released
942 from root-free soil samples (heterotrophic component) showed a variable range of ages, with
943 substantial components of pre-bomb carbon (carbon fixed before AD 1955). **C:** Detail of a
944 closed static chamber used to collect $CO_2$ from the soil efflux. Air is pumped in a closed loop
945 from the chamber through a quartz glass cartridge containing a zeolite molecular sieve, which
946 traps the $CO_2$ allowing it to be returned to the laboratory, recovered by heating, and analysed
947 by AMS.

948

## Box 3. Analysing rhizodeposit utilisation by microbes in the field

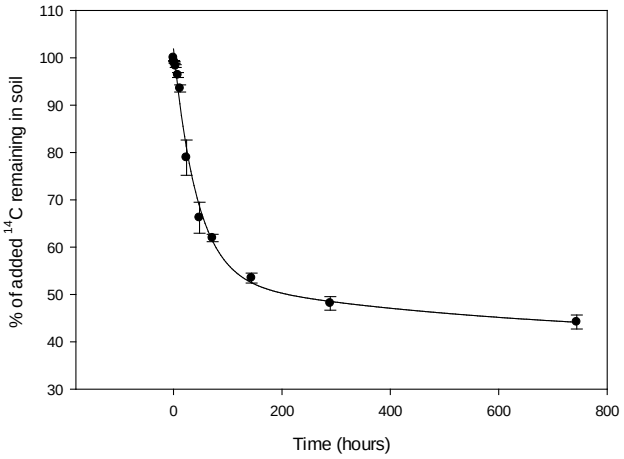


*In-situ* pulse-labelling experiments adding $^{13}C$-$CO_2$ to closed transparent chambers were used to study the translocation of the recently-fixed carbon belowground and the rhizodeposition utilization by microbes, e.g. by measuring $^{13}C$ incorporation into microbial biomarkers. **A:** At the Clocaenog site (UK-CL) this technique was applied along a peat layer gradient. Repeated pulses of $^{13}C$-$CO_2$ were applied during eight hours to *C. vulgaris* using sealed domes attached to a core inserted into the ground. **B:** The incorporation of $^{13}C$ into soil microbial PLFAs was analysed. Despite a high applied dose of $^{13}C$ (99 atom %), the dilution of the tracer within the large pool of unlabelled root biomass was remarkable, and as a consequence most of the analysed PLFAs showed no $^{13}C$ enrichment. **C:** $^{13}C$ recovery in Gram negative bacteria after a $^{13}C$-$CO_2$ pulse at the Brandbjerg site (DK-BRA). The enrichment pattern in PLFAs attributed to Gram negative bacteria in soils exposed to drought and elevated $CO_2$ concentration (+120

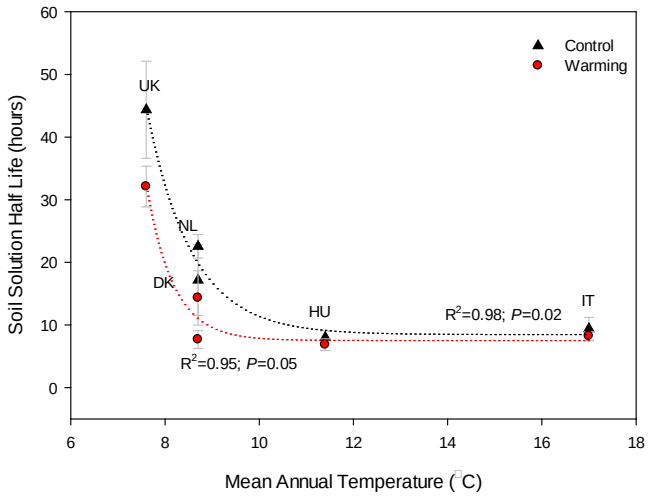
ppm) for 8 years show different carbon utilization patterns and magnitudes under imposed climatic conditions implying changed carbon cycle dynamics. **D:** Flow-through pulse-labelling equipment showing the gas reservoir containing $^{13}C$-$CO_2$ for up to eight hours of labelling connected to transparent Plexiglas chambers via tubing.

## Box 4. Exponential decay kinetics for $^{14}CO_2$ evolution during microbial $^{14}C$ substrate mineralisation

**A**




**B**




The catabolic utilisation profile, turnover and pool allocation of low molecular weight (LMW) carbon substrates was determined in soils collected across the experimental network. A selection of sixteen $^{14}C$ labelled amino acids and sugars varying in structural complexity and recalcitrance were used in a multiple substrate induced respiration (SIR) assay on soil. Evolved $CO_2$ was collected using NaOH traps and absorbed $^{14}CO_2$ was measured with a scintillation counter (Perkin Elmer). The rate at which radiolabelled substrates were metabolised by the microbial community was used to determine microbial uptake kinetics and turnover in the

absence of plants (no autotrophic input). A double term first order decay model with an asymptote described our data best;

$$f = y_0 + a_1 e^{-k_1 t} + a_2 e^{-k_2 t} \qquad \text{eqn. 1.}$$

where *f* describes the amount of $^{14}$C-labelled substrate or metabolites remaining in the soil at time *t*, the asymptote $y_0$ explains the $^{14}$C labelled material adsorbed to unavailable soil complexes or metabolites partitioned into unavailable recalcitrant material, the exponential coefficient $k_1$ describes the initial rapid uptake and respiration of $^{14}$C labelled substrate by the microbial community immediately following carbon substrate application, and $k_2$ describes a slower secondary mineralisation phase which we ascribed to immobilisation in microbial biomass and transformation of organic metabolites (microbial turnover). The terms $a_1$ and $a_2$ relate to the proportion of $^{14}$C labelled substrate that is associated with each exponential coefficient at time (*t*).

The mean residence time (MRT) or substrate half-life ($t_{1/2}$) can be calculated according to

$$t_{1/2} = \frac{\ln(2)}{k_n} \qquad \text{eqn. 2.}$$

The turnover of each pool can then be calculated as the inverse of the MRT (1/MRT).

**A:** For substrate mineralisation the equation: $y = 40.3766 + 48.3216^{0.0230x} + 13.1812^{0.0017x}$ fitted the data with an $r^2$ of 0.99. Using the coefficients ($k_n$) from the fitted equation, the half-life of the substrate can be calculated using eqn. 2. Substrate half-life was in the first phase (soil solution uptake) 30 h, and the second slower phase (microbial turnover) 408 h. Approximately 40 % of the substrate was immobilised in the soil, 48.3 % respired during the first phase, and 13.2 % respired during the slower second phase. **B:** Half-life of the substrate in the soil solution versus mean annual temperature, in control (triangle) and warming (circle) treatments, data points are mean ± SE (n=3). Warming treatment and relative warmer site, simply increases the catabolic utilisation of labile LMW-carbon until a threshold mean annual temperature of 11.5 $^{o}$C.