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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 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 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, including: 1) analysis of the naturally rare isotopes of carbon (^{13}C and ^{14}C) and nitrogen (^{15}N); 2) use of *in-situ* pulse labelling with $^{13}CO_2$, soil injections of ^{13}C- and ^{15}N-enriched substrates, or continuous labelling by Free Air Carbon dioxide Enrichment (FACE) and 3) manipulation of isotopic composition of soil substrates (^{14}C) in lab-based studies.

3. The natural ^{14}C signature of soil respiration gave insight into a possible long-term shift in the partitioning between the decomposition of young and 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. The use of labelled carbon-compounds to study carbon mineralization by soil microorganisms enabled to determine the long-term effect of climate change on microbial carbon uptake kinetics and turnover.

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 climate change experiments. ^{13}C-labelling techniques exert minimal physical disturbances,

however, the dilution of the applied isotopic signal can be challenging. In addition, the contamination of the field site with excess ^{13}C or ^{14}C can be a problem for subsequent natural abundance (^{14}C and ^{13}C) or label studies. The use of slight changes in carbon and nitrogen natural abundance does not present problems related to potential dilution or contamination risks, but the usefulness depends on the fractionation rate of the studied processes.

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

63 **Introduction**

64

65 Global climate change scenarios predict that increased greenhouse gas (e.g. CO_2, CH_4 and
66 N_2O) concentrations in the atmosphere will alter the periodicity and magnitude of drought
67 events and will increase mean global temperatures by approximately 0.2 °C per decade (IPCC
68 2013). For the European continent this will manifest as drier summers in the South and
69 increased precipitation in the North (IPCC 2013). Elucidating the consequences of such
70 atmospheric changes for biogenic carbon fluxes is one of the main challenges for the
71 scientific community. Some models have predicted a positive feedback to climate change,
72 resulting from higher increases in respiratory fluxes from ecosystems (e.g. carbon release
73 through soil respiration) than in net primary productivity, which would lead to further
74 increases in atmospheric CO_2 (Friedlingstein et al. 2006; Denman et al. 2007). To assess the
75 likelihood of this positive feedback, experimental studies that analyse the long-term
76 adaptations of ecosystem carbon fluxes to climate change are critically needed. However,
77 climate change experiments are often conducted at short or medium time scales due to
78 funding constraints, or due to the limited life-span of the experimental plots, as repeated
79 removal of samples often leads to disturbances and experimental artefacts in the studied
80 system. Hence, there is a necessity for the maintenance of long-term experiments using non-
81 destructive methods.

82 Carbon fluxes through the plant-soil-air continuum play a central role in soil carbon cycling
83 (Zak et al. 2000; Phillips et al. 2006). Consequently, aboveground to belowground fluxes
84 might largely determine carbon emissions from ecosystems under the different climate change
85 scenarios (Chapin et al. 2009). Stable carbon isotope studies can give important insights into
86 carbon fluxes through the plant-soil-air continuum with the minimal disturbance to the
87 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. 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 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 (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. 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, 2) trace experimentally-induced changes in the isotopic signatures to assess rhizodeposition utilisation by soil biota, and 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 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 (Fig. 1). 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. 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, including aboveground plant biomass (Kröel-Dulay et al. 2015), litter production, soil respiration and net ecosystem carbon exchange (Beier et al. 2008; Lellei-Kovács et al. 2016).

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 expressed as the delta (δ) notation (e.g. $\delta^{13}C$ and $\delta^{15}N$ in ‰), which is the deviation of the ^{13}C or ^{15}N abundance in the sample compared to a reference material (Brand & Coplen 2014). 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 (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.

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). 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). In general, an increase in the $\delta^{15}N$ 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 under a drought or warming could influence the leaf $\delta^{15}N$ as well (Michelsen et al. 1998; Andresen et al. 2009). For instance, the increase in plant $\delta^{15}N$ values with aridity may also result from increasing reliance on recycled organic N sources as opposed to new inputs.

Across the INCREASE network 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 manipulation. 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 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 (for a given common 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 and 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.

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

The natural radioactive ^{14}C abundance can be used to identify different sources of carbon in a mixed pool, for instance in soil respiration. Radiocarbon signatures of more recent (i.e. < 65-70 years) and older carbon sources 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' doubling at ca. 200% pMC). 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 its incorporation in plant biomass, the radiocarbon analysis of ecosystem fluxes found to contain bomb-^{14}C provides singularly unique information which crucially and directly confirms the 'recent' origin of any (decomposed) carbon substrate. 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, with the relative contribution of both sources of different ages being resolvable using a mixing model solution. Several studies have successfully achieved the separation of sources of C respiration across ecosystems using the 'bomb-^{14}C' method (Cisneros-Dozal 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 et al. 2009; Schuur et al. 2009). The method allows for a direct evaluation about possible differential effects of climate change factors on the fate of recent vs. older soil C moieties, a central question for climate change scientists. The applicability, sensitivity and accuracy of the method is obviously improved when more of the 'bomb-^{14}C' is detectable in the specific analysed C pool, e.g. containing relatively more C which laid down in living tissues and subsequent decomposition products in the 1950 to 1970s period.

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 (Domínguez et al. 2015, 2017). 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) 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

1. ^{13}C-CO_2 pulse labelling

In-situ pulse labelling with the stable carbon isotope (^{13}C) is a good method to address questions related to the time lag between carbon assimilation and CO_2 release from soil (Kuzyakov & Gavrichkova 2010). 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 photosynthetically active 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 if analysis of ^{13}C in specific compounds such as PLFA or RNA is performed. 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 (Treonis et al., 2004; Rangel-Castro et al. 2005), with a maximum incorporation of ^{13}C into microbial RNA or biomass occurring within one to eight days after the pulse (Ostle et al. 2003; Butler et al., 2004). These studies have also shown that this flux 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 (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).

In the INCREASE network, several pulse-labelling experiments were 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). Transparent domes of 50 cm diameter and 100 cm height, enclosing individual *C. vulgaris* plants, were used. Repeated pulses of ^{13}C-CO_2 (99 atom% ^{13}C = 99% ^{13}C + 1% ^{12}C) were applied 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 $\leq$ 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. From these labelling experiments we learned that climate change factors change the flow of carbon within the plant-soil-atmosphere continuum. Increased atmospheric CO_2 concentrations accelerate the carbon cycle as seen as labelled carbon through the bacterial community over time. In contrast, drought slowed down carbon transport dynamics with soil microbes showing the ^{13}C label later in time (Reinsch et al. 2014).

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

337 increase if the labelling period is prolonged, which also affects photosynthetic processes
338 (Epron et al. 2012). Losses of ^{13}C due to physical diffusion and adsorption/desorption into the
339 chamber and tubing material should also be considered. In particular, the back-diffusion of the
340 $^{13}CO_2$ from the soil to the atmosphere which entered the soil pores during the labelling might
341 confound the interpretation of measured belowground respiration (Subke et al. 2009; Selsted
342 et al. 2011). However, when applied properly, the insights into terrestrial carbon allocation
343 can be detailed and novel (Box 3).
344

345 ***2. Free Air Carbon dioxide Enrichment (FACE)-labelling***
346

347 An alternative method for ^{13}C labelling of vegetation and whole-ecosystems is to use ^{13}C-
348 depleted CO_2 in FACE experiments. The FACE technique has through decades been used
349 within cropping systems (Kimball 2016), grasslands (Hovenden et al. 2014; Reich et al. 2014;
350 Mueller et al. 2016) and forests (Terrer et al. 2016) experiments, with the primary goal of
351 assessing potential carbon dynamics and enhancement of plant growth (Andresen et al. 2016).
352 As a side effect, the change in carbon isotopic composition of vegetation exposed to the
353 FACE-treatment can be used to trace freshly assimilated carbon into soil microbial biomass,
354 fauna and organic carbon pools. This approach was used at the Brandbjerg site (DK-BRA).
355 The CO_2 used to elevate concentrations of atmospheric CO_2 to 510 ppm had $\delta^{13}C$ values
356 ranging from –3.0 to –36.7 ‰ throughout 8 years of experimental treatment, with an overall
357 mean of -26.1 ‰. The source of the CO_2 supplied was brewery surplus CO_2 as a chemically
358 obtained side product. The mixing of the added CO_2 via FACE with ambient CO_2 in the
359 moving air mass resulted in a ^{13}C depletion ranging from -6.7 to -15.6 ‰. On average, this
360 equals a depletion of CO_2 in FACE plots of -4.8 ‰ relative to the atmospheric -8 ‰ average.
361 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 a 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 in each PLFA biomarker, also illustrating the pathway of newly-assimilated carbon into microbial biomass

A general drawback of the ^{13}C-FACE label is again the 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. Also, FACE-CO_2 can only be used as tracer if the isotopic composition of the FACE-CO_2 is considerably different than the isotopic composition of the atmospheric CO_2.

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

As a much more localized approach, *in-situ* injection 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) (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 and later used for PLFA extractions. The ^{13}C enrichment in PLFA

412 markers thus indicated the activity (vitality) of the specific microbial group (Watzinger 2015).
413 We found that bacteria opportunistically utilised the freshly added glycine substrate, i.e.
414 incorporated ^{13}C, whereas fungi showed only minor or no glycine derived ^{13}C-enrichment
415 (Andresen et al. 2014). In comparison, ^{13}C traced into the microbial community via the ^{13}C-
416 CO_2 pulse label at the same site (DK-BRA) also reached the bacterial community first.
417 Bacteria showed high ^{13}C enrichment compared to fungal groups (Reinsch et al. 2014). This
418 suggests that *in-situ* injection of ^{13}C substrates might be a plausible alternative to mimic
419 rhizodeposition effects. With the direct addition of ^{13}C label to the soil a strong labelling of
420 the microbial community was more easily achieved than with the indirect ^{13}C labelling of
421 microbes via plant assimilated ^{13}C-CO_2 (Box 3).
422

423 **Use of labelled carbon-compounds to analyse carbon mineralisation by soil**
424 **microorganisms**
425

426 Since soil microorganisms have an important role in controlling the availability of nutrients
427 via mineralisation of SOM, our understanding of how microbial functioning in the ecosystem
428 is altered by global change must be improved (Grayston et al. 1997). Microbial catabolic
429 diversity of a soil is directly related to the carbon decomposition function within a soil and
430 potentially provides a sensitive and ecologically relevant measure of the microbial community
431 functional structure (Garland & Mills 1991). Subsequently, multiple assays have been
432 developed to generate community level physiological profiles (CLPP) that can act as
433 fingerprints of microbial function. Three approaches for measuring CLPP in soils are reported
434 in the literature: (i) Biolog (Garland & Mills 1991); (ii) a substrate-induced respiration (SIR)
435 technique (Degens & Harris 1997); and (iii) MicroResp (Campbell et al. 2003). These
436 methods are all based on quantifying CO_2 respired during the mineralisation of organic carbon

437 compounds that vary in size, charge and structural complexity. The first approach, Biolog
438 MicroPlateTM (Biolog), assesses the catabolic diversity of soil organisms using a microtitre
439 plate by incubating a soil culture in the presence of nutrients and 95 different carbon
440 substrates; respired CO_2 is used to reduce a tetrazolium violet salt, which results in a colour
441 change that can be quantified colorimetrically (Garland & Mills 1991). This approach,
442 however, has been criticized for bias towards fast growing organisms that thrive in culture
443 (Preston-Mafham et al. 2002). In response to the criticism of the Biolog method, Degens &
444 Harris (1997) developed a method based on SIR where individual substrates are added to
445 intact soil and evolved CO_2 is sampled and quantified. Finally, Campbell et al. (2003)
446 combined aspects of both methods (MicroRespTM) where the response to carbon substrate
447 addition to soil is measured colorimetrically using a cresol red indicator dye in a microtitre
448 plate format.

449 Community level physiological profiling of soils samples collected from all treatments across
450 the network was conducted to determine the catabolic utilisation profile, turnover and pool
451 allocation of low molecular weight (LMW) carbon compounds by using a selection of ^{14}C-
452 labelled substrates. This method enabled the attribution of respired CO_2 to specific metabolic
453 processes that facilitates the quantification and qualification of microbial mineralisation
454 kinetics of substrates varying in structural complexity and recalcitrance. The kinetics of
455 microbial ^{14}C-CO_2 evolution can be described using a first order exponential decay model
456 (Box 4). The number of terms used in the exponential decay model can be used to explain
457 how microbial kinetics relates time, substrate complexity and carbon pool allocation to, for
458 example, rapidly cycled labile soil solution carbon, microbial structural carbon and
459 recalcitrant extracellular soil organic carbon (Kuzyakov & Demin 1998; Nguyen & Guckert
460 2001; Boddy et al. 2007). Attribution of modelled carbon pool sizes and turnover rates to
461 biological function are not only time and substrate dependent. Therefore, soil physical,

462 biological and chemical interactions may be miss-attributed to biological function. Indeed,
463 current knowledge and techniques available might not be enough to examine the interaction
464 between discrete carbon pools (Glanville et al, 2016). Using the half-life of ^{14}C labelled
465 carbon in soil solution we were able to examine the environmental gradient of the warming
466 treatment across the climate change network and identified that temperature becomes rate
467 limiting for microbial uptake of carbon from the soil solution pool at < 10.5 °C. We also
468 showed that experimentally manipulated warming simply speeds up the catabolic utilisation
469 of labile LMW carbon in a predictable pattern (Box 4).
470

471 **Conclusions and recommendations**
472

473 Stable isotope studies provide insightful information about carbon (and nitrogen) fluxes
474 through the plant-soil-atmosphere continuum with minimal disturbance to the system. The
475 value of the different isotope techniques depends on the specific research questions.

476 The analysis of the natural abundance of the heavy isotopes is only useful when isotope
477 signatures in the different carbon or nitrogen pools are clearly distinct as a result of important
478 fractionation processes. In practice, the application of this technique is limited to the study of
479 the effects of changing abiotic conditions on processes that operate over a relative broad
480 period of time, for instance to study changes in plant water use efficiency or N sources in a
481 drought experiment over the growing season or different years. In contrast, the radiocarbon
482 analysis ("bomb-C" technique) of instantaneous fluxes (soil or ecosystem respiration) has
483 been proved to be very useful to evaluate whether different factors of climate change provoke
484 the release of older carbon sources through soil or ecosystem respiration, a central question in
485 relation to the proposed positive feedback between climate change and SOM decomposition.

However, the progressive dilution of the bomb-C signature of the atmosphere will limit the application of this technique in the upcoming decades.

If the analysis of climate change effects on plant carbon belowground and cycling through the microbial community is the main research interest, then ^{13}C labelling approaches are the most appropriated tools. Coupled with the analysis of ^{13}C in specific microbial compounds, this technique constituted a remarkable advance in the study of processes occurring at the rhizosphere level. A significant challenge of the application of this technique is the achievement of sufficient ^{13}C enrichment in microbial biomass where the pools of background carbon in the studied compartments are high and hence dilute the ^{13}C signal. As an alternative, direct injection of ^{13}C-enriched substrates into soil can be applied to mimic rhizodeposition and to achieve a higher ^{13}C signal in the microbial community. Fumigation with FACE-CO_2 can be used to achieve a longer-term labelling of soil microbes and fauna.

The application of these techniques, however, 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.

For *in-situ* pulse-labelling studies 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. 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' method is very sensitive to the

510 contamination of soil or plant samples with ^{14}C-enriched material, and thus its application
511 should be limited to sites and facilities where no ^{14}C-labelling work has been conducted.
512 Additionally, it should be noted that any history of fertilization might also alter the natural
513 isotope abundance of ecosystem compartments (in particular ^{15}N signatures), potentially
514 confounding experimental results. The surroundings of a FACE experiment can be also
515 "contaminated" by draft winds carrying the depleted label onto ambient plots.
516

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529

530 **Data Accessibility**

531 The manuscript does not have associated archived data.
532

533 **Author contributions statement**

All authors contributed to the collection of the data included as illustration of the methodologies. LCA, MTD, SR, and ARS wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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780 **Table 1**. Suggestions and advice to consider when applying isotopic methods for the study of carbon fluxes in the plant-soil system.
781

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 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. - 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). - 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. - 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.	- If you are not sure about potential ^{14}C contamination in your lab, use another lab or make a swipe test. - Make previous trials to assess the incubation times required to get a sufficient CO_2 sample - 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-CO_2 pulse-labelling	^{13}C- enriched compounds used for labelling and as standards are usually expensive; ^{13}C determination in specific compounds is expensive, although cheaper than AMS	- Consider the target pools to be analysed and the potential dilution of the label by the unlabelled root system or soil carbon pool. - If your study requires a high ^{13}C enrichment, mind the potential risk of contaminating the site. - Avoid above ambient CO_2 concentrations in the chamber. - If you need to monitor CO_2 during your pulse, remember that IRGAs are rather insensitive to $^{13}CO_2$.	- 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). - 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 immediately after the pulse). - Experiments requiring root washing or microbial compound extraction are time consuming.
Natural abundance of isotopes (^{13}C and ^{15}N)	IRMS analysis is relatively cheap	- Make sure the history of sampling site is known (previous labelling experiments?)	- Be aware that FACE can dilute the isotopic signal, most CO_2 enriched systems use ^{13}C depleted		- Sampling time and grinding / weighing of sample. - Analysis usually

			sources,because this is cheaper.	done at a dedicated natural abundance facility.
^{14}C-substrates mineralisation	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*	Similar to ^{13}C-CO_2 pulse-labelling.	- Contamination risk of ^{13}C leaching is present, but smaller to our judgement than from ^{13}C-CO_2 experiments. - 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. - Sample handling from field work until the extraction takes a few days so plan only one sampling event per week if possible.

784 **Figure Captions**

785

786 **Figure 1**. Map of the European INCREASE network, with the shrubland field sites and
787 annual temperature (red line, right axis) and precipitation (bars, left axis) norm. Sites in
788 Denmark: Mols (DK-MOLS) and Brandbjerg (DK-BRA); in United Kingdom: Clocaenog
789 (UK-CL) and Peaknaze (UK-PK); in The Netherlands (NL): Oldebroek; in Spain (SP):
790 Garraf; in Italy (IT): Porte Conte, and in Hungary (HU): Kiskunsàg.

791

792 **Box 1.** Isotopic signal of plant leaf responses to precipitation. Stable isotopes ($\delta^{13}C$ and $\delta^{15}N$)
793 in aboveground plant material collected across the network was analysed by isotopic ratio
794 mass spectrometry (IRMS). A: Leaves and twigs (t) from *P. alba* (HU), *E. multiflora L.* (SP),
795 *G. alypum L.* (SP) and *C. vulgaris* (NL); filled circle ● is control, open circle ○ is drought
796 treatment, ▼ is warming treatment. P-values indicate effects of treatment, year, and the
797 interaction of these factors on ^{13}C or ^{15}N, analysed by two-way ANOVA; ns is non-significant
798 effect. Number indicates year (2001=1, 2002=2, 2003=3 or 2004=4). Species (site)
799 differences and annual differences are stronger than treatment effects. B: $\delta^{13}C$ of *C. vulgaris*
800 leaves versus annual precipitation of the previous year.
801

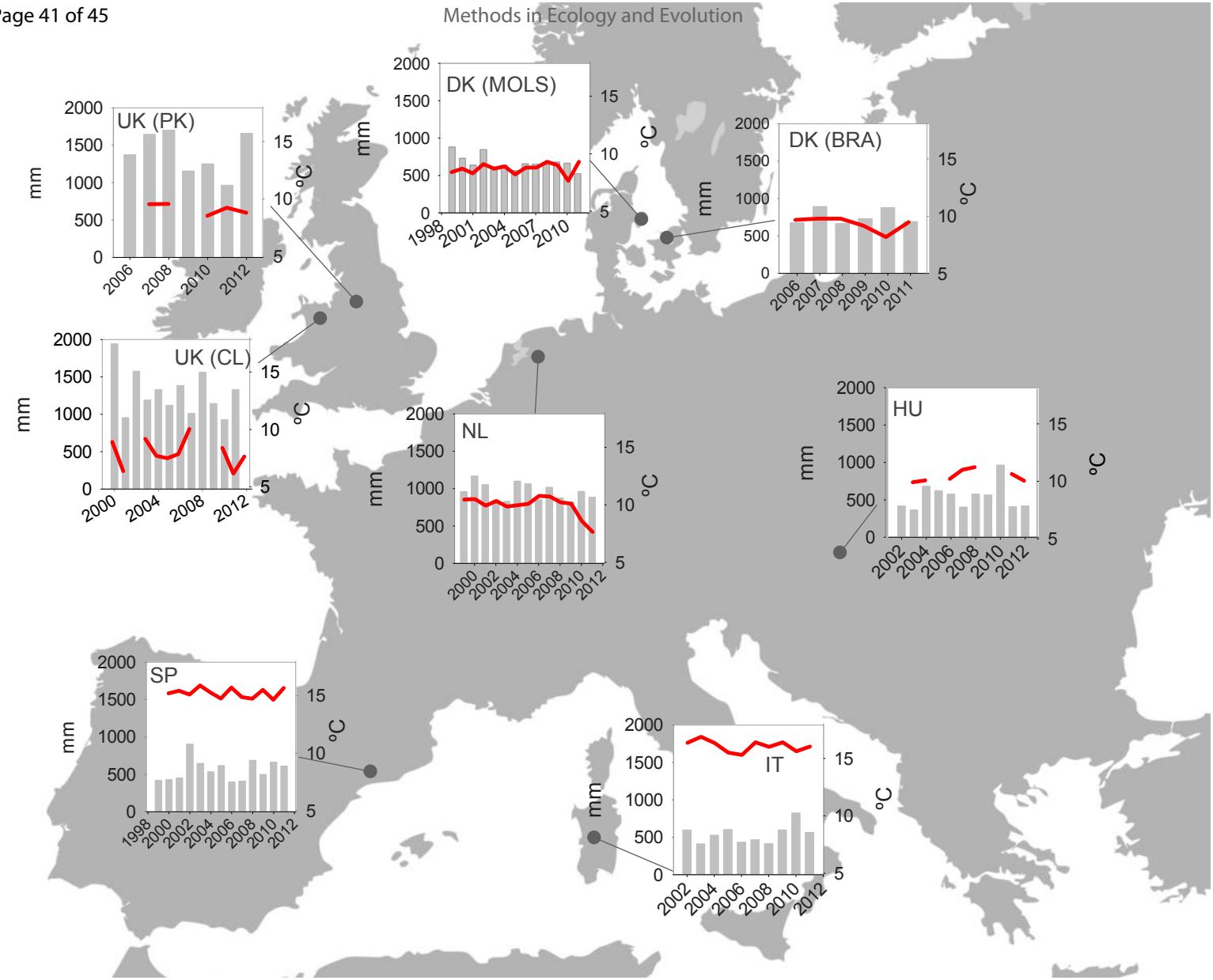
802 **Box 2.** Impact of warming and drought on the ^{14}C signature of soil respiration. **A:** Records of
803 atmospheric ^{14}C over the 20th century. The unit for ^{14}C signature (% Modern) is a
804 measurement of the deviation of the $^{14}C/^{12}C$ ratio of a sample from the "Modern" standard,
805 which is defined as 95% of the radiocarbon concentration (in AD 1950) of a reference
806 material (NBS Oxalic Acid I, SRM 4990B), adjusted to a $\delta^{13}C$ reference value of –19 ‰. **B:**
807 At the UK-PK site, the ^{14}C signature of the soil efflux was measured (bars, left axis). ^{14}C
808 values were highly heterogeneous (ranging from 105.49 to 110.13 % Modern; values of > 100
809 % Modern suggest that a substantial component -and potentially all- of the carbon was
810 trapped by photosynthesis during the post-bomb era i.e. since ~AD 1955).There were no
811 significant effects of the warming treatment, while there was a trend towards the release of
812 older carbon in the drought plots. On average, the carbon being released from the plots had
813 been fixed from the atmosphere between six and eight years earlier (line, right axis). **C:** Detail
814 of a closed static chamber used to collect CO_2 from the soil efflux.

815

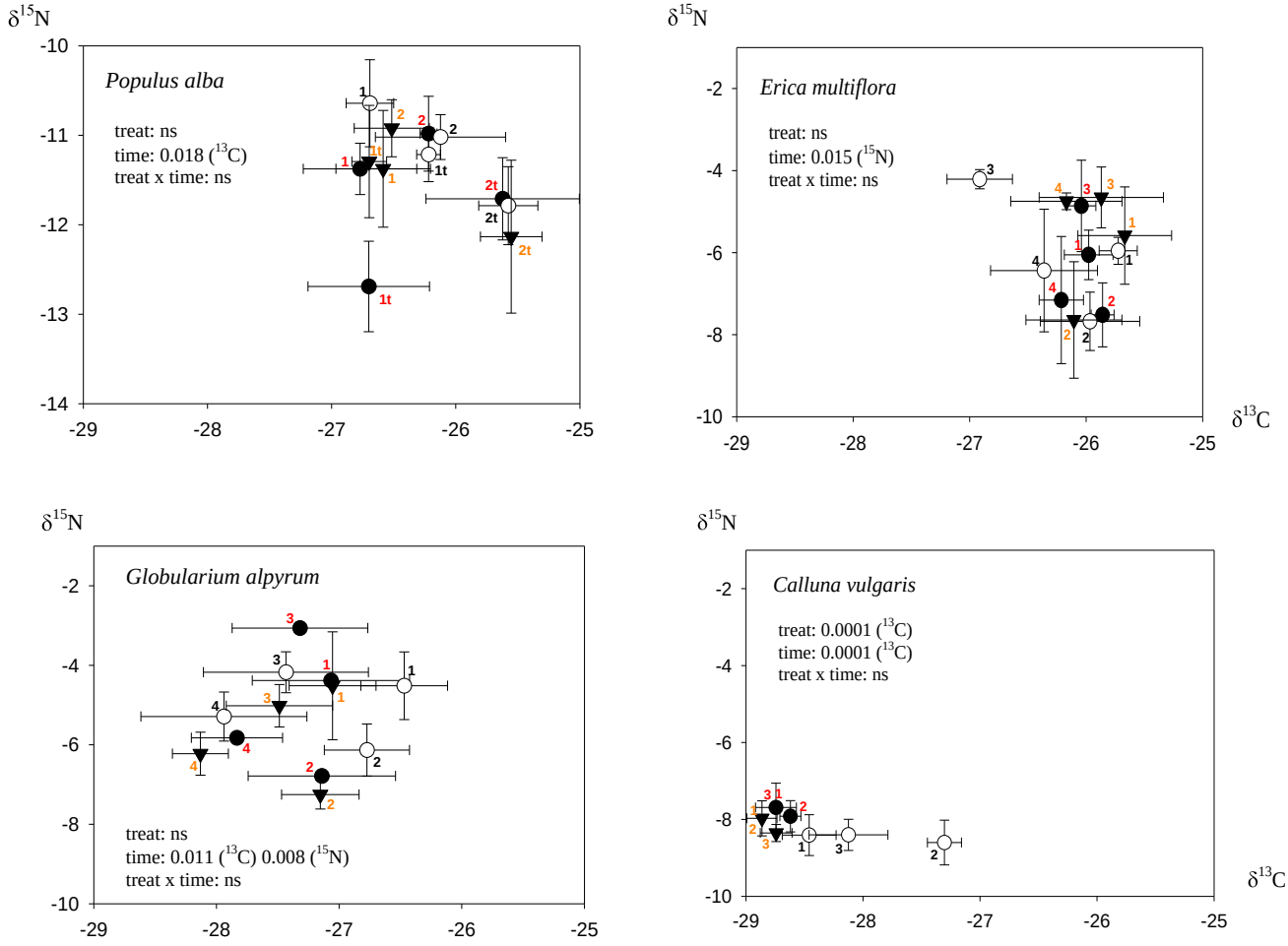
816 **Box 3**. Analysis of rhizodeposit utilisation by microbes using *in-situ* ^{13}C-CO_2 pulse-labelling
817 experiments. **A:** At the Clocaenog site (UK-CL) this technique was applied along a peat layer
818 gradient. Repeated pulses of ^{13}C-CO_2 were applied during eight hours to *C. vulgaris* using
819 sealed transparent domes attached to a core inserted into the ground. **B:** The incorporation of
820 ^{13}C into soil microbial PLFAs was analysed. Despite a high applied dose of ^{13}C (99 atom %),
821 the dilution of the tracer within the large pool of unlabelled root biomass was remarkable, and
822 as a consequence most of the analysed PLFAs showed no ^{13}C enrichment. **C:** ^{13}C recovery in
823 Gram negative bacteria after a ^{13}C-CO_2 pulse at the Brandbjerg site (DK-BRA). The
824 enrichment pattern in PLFAs attributed to Gram negative bacteria in soils exposed to drought
825 and elevated CO_2 concentration (+120 ppm) for 8 years show different carbon utilization
826 patterns and magnitudes under imposed climatic conditions implying changed carbon cycle
827 dynamics. **D:** Flow-through pulse-labelling equipment showing the gas reservoir containing
828 ^{13}C-CO_2 for up to eight hours of labelling connected to transparent Plexiglas chambers via
829 tubing.
830

831 **Box 4**. Exponential decay kinetics for $^{14}CO_2$ evolution during microbial ^{14}C substrate
832 mineralisation**.** The catabolic utilisation profile, turnover and pool allocation of low molecular
833 weight (LMW) carbon substrates was determined in soils collected across the experimental
834 network. Sixteen ^{14}C labelled amino acids and sugars varying in structural complexity and
835 recalcitrance were used in a multiple substrate induced respiration (SIR) assay on soil.
836 Evolved CO_2 was collected using NaOH traps and absorbed $^{14}CO_2$ was measured with a
837 scintillation counter. **A:** For substrate mineralisation a double-term first order decay model
838 with an asymptote fitted the data with an r^2 of 0.99. Using the coefficients from the fitted
839 equation, estimated half-life of the substrate in the first phase (soil solution uptake) was 30 h,
840 and in the second slower phase (microbial turnover) 408 h. Approximately 40 % of the
841 substrate was immobilised in the soil, 48.3 % respired during the first phase, and 13.2 %
842 respired during the slower second phase. **B:** Half-life of the substrate in the soil solution
843 versus mean annual temperature, in control (triangle) and warming (circle) treatments, data
844 points are mean ± SE (n=3). Warming treatment and relative warmer site, simply increases
845 the catabolic utilisation of labile LMW-carbon until a threshold mean annual temperature of
846 11.5 ºC.

847

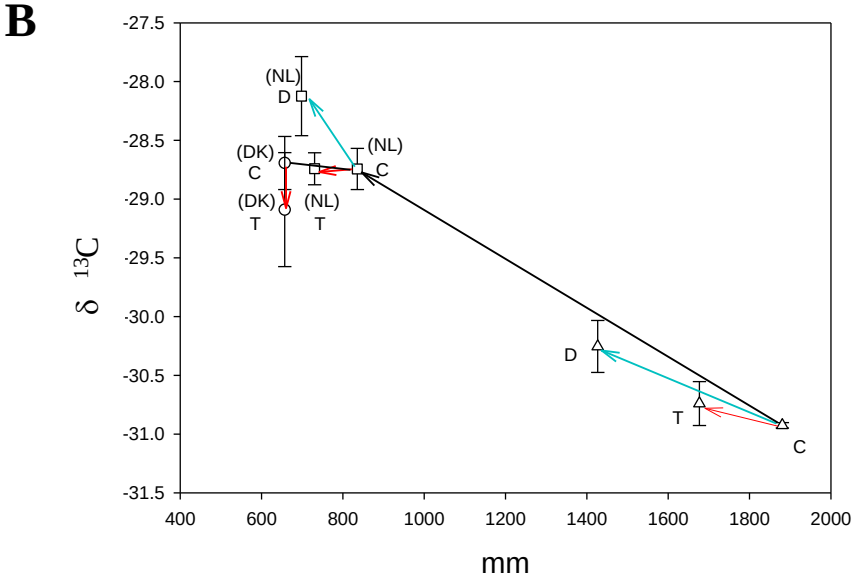

UK (PK)
DK (MOLS)
DK (BRA)
UK (CL)
NL
HU
SP
IT
mm
°C
2000
1500
1000
500
0
15
10
5
2006
2008
2010
2012
1998
2001
2004
2007
2011
2000
2002
2012

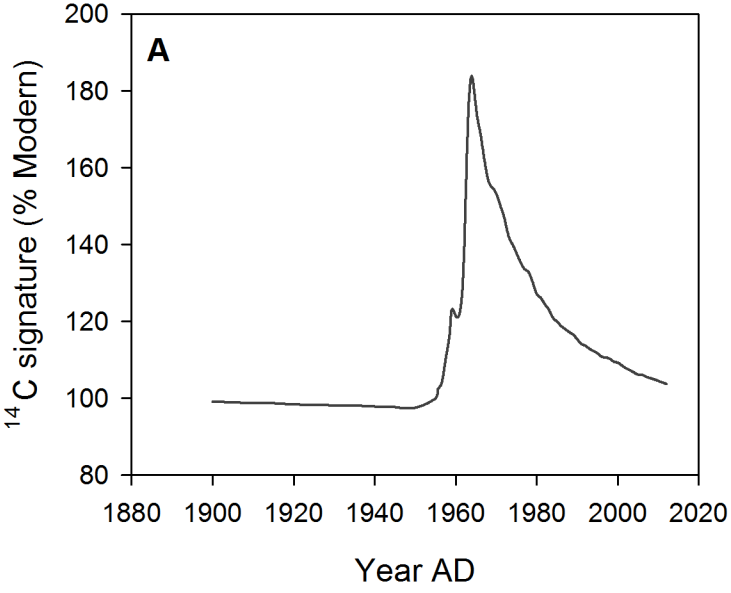
A

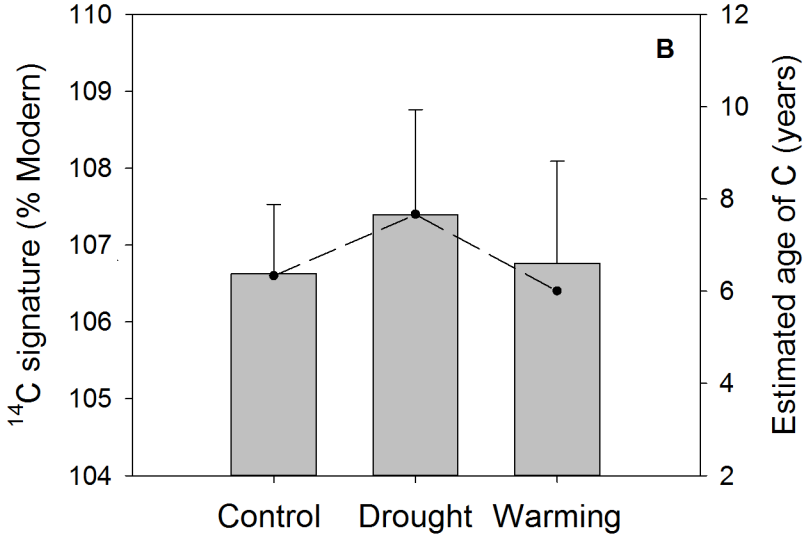


δ13C vs precipitation previous year

B

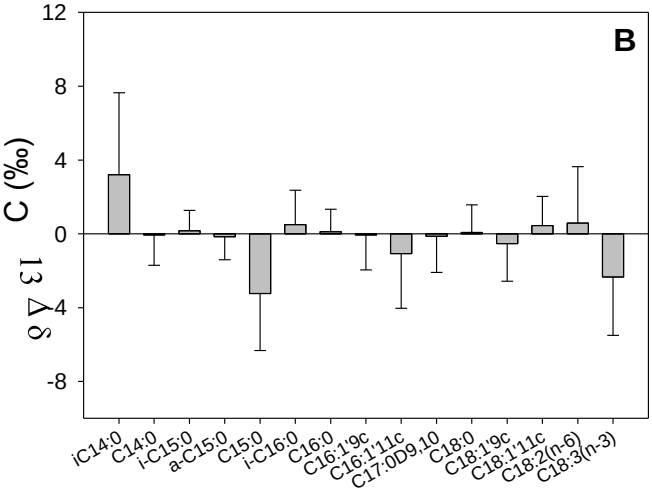


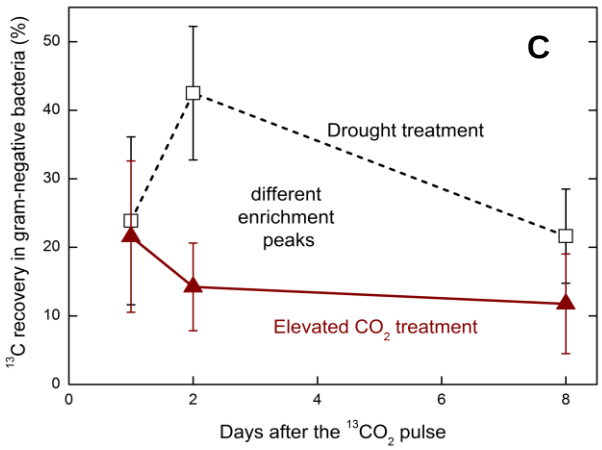

A
200
180
160
140
120
100
80
^{14}C signature (% Modern)
1880
1900
1920
1940
1960
1980
2000
2020
Year AD


B
110
109
108
107
106
105
104
^{14}C signature (% Modern)
12
10
8
6
4
2
Estimated age of C (years)
Control
Drought
Warming

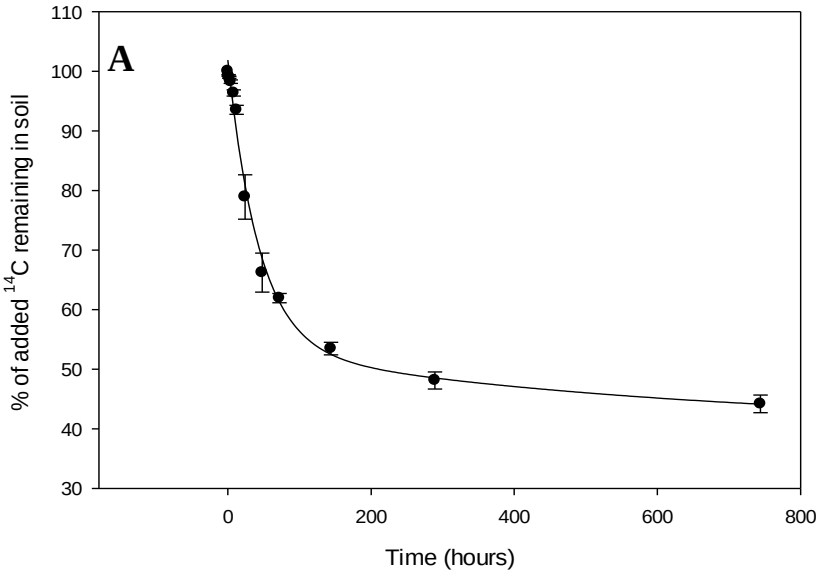

C

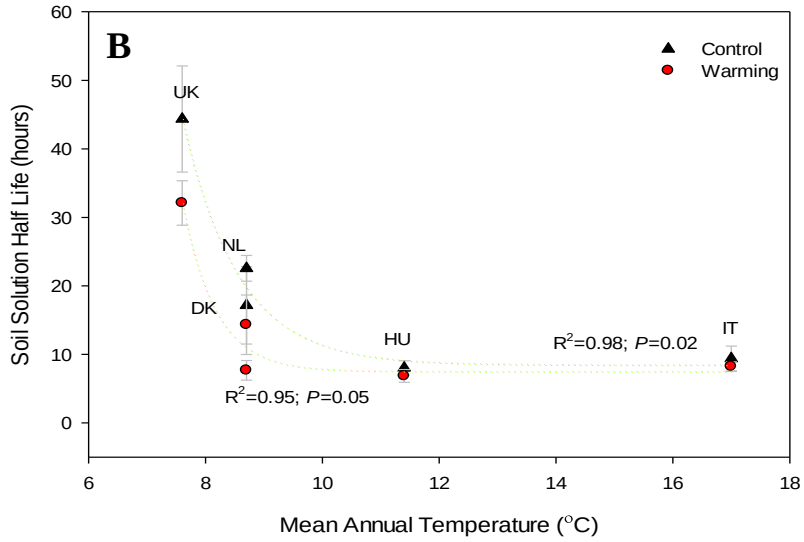

A


B
12
8
4
0
-4
-8
$\Delta\delta^{13}C$ (‰)
iC14:0
C14:0
i-C15:0
a-C15:0
C15:0
i-C16:0
C16:0
C16:1'9c
C16:1'11c
C17:0D9,10
C18:0
C18:1'9c
C18:1'11c
C18:2(n-6)
C18:3(n-3)


C
^{13}C recovery in gram-negative bacteria (%)
50
40
30
20
10
0
0
2
4
6
8
Days after the $^{13}CO_2$ pulse
Drought treatment
different
enrichment
peaks
Elevated CO_2 treatment


D


A
% of added ^{14}C remaining in soil
Time (hours)
110
100
90
80
70
60
50
40
30
0
200
400
600
800


B
Control
Warming
UK
NL
DK
HU
IT
R^2=0.98; P=0.02
R^2=0.95; P=0.05
Soil Solution Half Life (hours)
Mean Annual Temperature (°C)
60
50
40
30
20
10
0
6
8
10
12
14
16
18