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Methods paper

Improved methodology for tracing a pulse of ^{13}C-labelled tree photosynthate carbon to ectomycorrhizal roots, other soil biota and soil processes in the field

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Isotopic pulse-labelling of photosynthate allows tracing of carbon (C) from tree canopies to below-ground biota and calculations of its turnover in roots and recipient soil microorganisms. A high concentration of label is desirable but is difficult to achieve in field studies of intact ecosystem patches with trees. Moreover, root systems of trees overlap considerably in most forests, which requires a large labelled area to minimize the impact of C allocated below-ground by un-labelled trees. We describe a method which combines a high level of labelling at ambient concentrations of CO_2, [CO_2], with undisturbed root systems and a model to account for root C and root-derived C from un-labelled trees. We raised 5-m-tall chambers, each covering 50 m^2 of ground (volume 250 m^3) in a young boreal *Pinus sylvestris* L. forest with up to 5 m tall trees. Rather than a conventional single release of $^{13}CO_2$, we used five consecutive releases, each followed by a draw-down period, thus avoiding high [CO_2]. Hence, we elevated successively the $^{13}CO_2$ from 1.1 to 23 atom% after the first release to 61 atom% after the fifth, while maintaining [CO_2] below 500 p.p.m. during 4–4.5 h of labelling. The average abundance of $^{13}CO_2$ was as high as 42 atom%. We used the central 10 m^2 of the 50 m^2 area for sampling of roots and other soil biota. We modelled the dilution of labelled C across the plots by un-labelled C from roots of trees outside the area. In the central 10 m^2 area, ~85% of roots and root-associated biota received C from labelled trees. In summary, we elevated the labelling of roots and associated soil biota four-fold compared with previous studies and described the commonly overlooked impact of roots from un-labelled trees outside the labelled area.

Keywords: forests, mycorrhiza, soil biology, stable isotope probing, tree below-ground C flux.

Introduction

Tree photosynthesis feeds soil biota with carbon (C) through aboveground litter-fall and a roughly equally large below-ground flux to roots and associated organisms, notably mycorrhizal fungi (Hanson et al. 2000, Högberg and Read 2006). Insight into the quantitative role of plant below-ground C flux to specific soil organisms and soil processes requires isotope tracer studies, which are challenging to perform in the field due to the size of trees (Epron et al. 2012). The pioneers Horwath et al. (1994) used radioactive ^{14}C, an approach further developed by Carbone et al. (2007).

Advancements in isotope ratio mass spectrometry (IRMS) and wave-length scanner cavity ring-down spectroscopy have promoted the use of stable ^{13}C, with no need to consider radiation safety. Using ^{13}C makes it possible to use elaborate laboratory methods targeting a range of soil organisms. High tracer levels have enabled labelling of phospholipid fatty acid (PLFA) biomarkers of specific groups of soil microorganisms, their RNA, DNA and other macromolecules in laboratory settings or in field studies of low-stature plants (e.g., Radajewski et al. 2000, Treonis et al. 2004, Vandenkoornhuyse et al. 2007, Pett-Ridge and Firestone 2017, Kleiner et al. 2023). Short-term labelling, pulse-labelling, followed by frequent sequential sampling of target tree organs and soil biota enables calculations of C turnover rates (e.g., Högberg et al. 2010, Keel et al. 2012).

Studies of plant mesocosms or of small plants in the field can use more elaborate designs for labelling plants and tracing plant photosynthate into soils (e.g., Epron et al. 2012) than studies of trees at the ecosystem patch scale. This scale is desirable in studies of ecosystem C budgets and for realistic predictions of interactions among trees and between trees and soil biota, but it is much more costly and faces special technical challenges. The prime challenge in pulse-labelling studies is to achieve labelling high above variations in natural abundance of ^{13}C in trees and in recipient soil organisms, processes and compounds of interest. Furthermore, since $^{13}CO_2$ is expensive, as much of it as possible should be

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assimilated. A second important challenge is to ensure that the belowground system studied is not disturbed and reflects the natural connection with the labelled tree canopy, i.e., that area-based budget estimates of above- and below-ground C are correct.

Two different approaches are used for pulse-labelling of trees with $^{13}CO_2$ in field settings (Epron et al. 2012). The single-tree method uses a chamber enclosing the tree crown which is sealed around the lower part of the stem (e.g., Plain et al. 2009, Gao et al. 2021). It is ideal for providing data from replicate trees, especially regarding above-ground processes. For studying the flux of C to below-ground components in the field, chambers enclosing several trees (thus representing an ecosystem patch) are more appropriate (Högberg et al. 2008, 2010). However, this also means that large chambers are needed, especially where tree root systems overlap considerably, as is common (Stone and Kalisz 1991).

We have previously pulse-labelled 50 m^2 patches of young boreal *Pinus sylvestris* L. forest with 4 to 5 m tall trees (Högberg et al. 2008, 2010). In such studies, the air in the chambers used for labelling is open to the respiratory efflux from roots and other soil biota. At this scale, it is not feasible to keep the concentration of CO_2, [CO_2], or the atom% ^{13}C constant, nor are these constant under natural conditions. One cannot effectively scrub away the large background of $^{12}CO_2$ to replace it with $^{13}CO_2$ because the respiratory efflux will continuously add new un-labelled CO_2, especially from the soil (Subke et al. 2009). Thus, both respiration and tracer additions will add CO_2 into chamber air, while uptake through photosynthesis will remove it. As a result, the chamber air [CO_2] will change depending on the balance between these processes. Nevertheless, it is desirable to keep the [CO_2] within a reasonable range in relation to naturally occurring levels and variations.

We have earlier used a single release of $^{13}CO_2$ and 1.5–3.5 h long incubations (e.g., Högberg et al. 2010). Here, we report a method to substantially increase the level of labelling of CO_2 in chambers by making five consecutive releases of $^{13}CO_2$ during periods of 4 to 4.5 h while maintaining the [CO_2] within a reasonable range. Thus, we avoided high levels that would approach or even exceed the A_{max} of photosynthesis and potentially alter the C allocation patterns in the studied system. We compare the levels of labelling in below-ground components and fluxes in this study with those obtained previously using a single release of $^{13}CO_2$ in a full-scale study conducted in 2007 (Högberg et al. 2010, Keel et al. 2012) and in a pilot study made in 2006 (Högberg et al. 2008). Based on the amounts of $^{13}CO_2$ added and a change in chamber volume, we predicted a four fold increase in labels in target organisms and processes between 2007 and 2012.

In studies of this kind, it is often overlooked that roots of un-labelled trees occur under the canopy of the labelled tree or labelled group of trees. This neglect is based on the assumption that the distribution of the root system of a tree can be predicted as vertical projections of its crown, which is not correct since root systems often overlap considerably (e.g., Stone and Kalisz 1991). Ignorance of this fact results in a mismatch between the above- and below-ground C budgets in studies employing $^{13}CO_2$ labelling. Using data on the horizontal extent of tree roots, we elaborate on how the influence from roots of un-labelled trees outside the chambers varies depending on the size of the chambers used.

Table 1. Characteristics of the two *P. sylvestris* stands we use in our comparison, Rosinedalsheden (Högberg et al. 2008, Högberg et al. 2010) and Åheden (this study). The number of plots was one for the pilot study at Rosinedalsheden (Högberg et al. 2008), but eight for the full-scale study at the same site (Högberg et al. 2010) as compared with two at Åheden. Data on NEE at Rosinedalsheden are from Metcalfe et al. (2017). All other data from Rosinedalsheden are from Högberg et al. (2010).

Parameter	Rosinedalsheden	Åheden
Tree age (years)	14	15
Tree height (m)	2.5	3.3
Soil C/N ratio	33	37
Soil pH	4.5	4.4
Ecosystem NEE (g CO_2 m^{-2} h^{-1})	1.1	1.2

NEE in summer at mid-day at 475 p.p.m. CO_2.

Materials and methods

Site studied

We studied a young, naturally regenerated boreal *P. sylvestris* L. forest (Table 1) also studied by Hasselquist et al. (2016). It is located 60 km NW of Umeå, Sweden, at Åheden (64°14′N, 19°46′E, at 175 m a.s.l.). The soil is podzolized coarse silt. It has a 1–3 cm thick organic mor-layer with a C:N ratio of 37 and a pH_{H2O} of 4.4. Trees were, on average, 3.3 m tall and had a diameter at breast height of ∼4 cm; larger trees were close to 5 m tall. Some of the trees had cones and were, in that respect, mature.

Method of labelling

We established three plots in late July 2012. For ^{13}C labelling, we later raised on two of the plots 5 m tall octagon-shaped plastic chambers, each covering 50 m^2 patches of the forest ecosystem. Thus, each chamber contained a volume of 250 m^3. Their design, temperature control, circulation of air, etc., were described in detail in Högberg et al. (2008) and Högberg et al. (2010). The third plot was a control plot not used for labelling but for obtaining measures of background variations in the natural abundance of ^{13}C. Such measures are important in studies using a low level of labelling but much less so when a high level of labelling is used. Of the two plots to be ^{13}C-labelled, one was treated with nitrogen (N) by adding the equivalent of 150 kg N ha^{-1} as $Ca(NO_3)_2$ in the form of pellets on 27 July, i.e., 3 weeks before the ^{13}C labelling. This had the purpose of comparing the effects of N on ^{13}C distribution with those in previous studies (Högberg et al. 2010, Näsholm et al. 2013). However, in these studies, effects of N on C allocation were not observed in the short term (first month) but were profound after a year (Högberg et al. 2010). Given the lack of immediate effects of N on below-ground C allocation, we here used the ^{13}C-labelled N plot as a replicate of the ^{13}C-labelled plot.

In the pilot study in 2006, we used a single release of 5 L of $^{13}CO_2$ at ≥95 atom% ^{13}C, which resulted in 3.7 atom% $^{13}CO_2$ in the chamber air and a [CO_2] of ∼360 parts per million (p.p.m. or μmol mol^{-1}) directly after the release (Högberg et al. 2008). In the full-scale study in 2007, we used a single release of 25 L of $^{13}CO_2$ at 99 atom% ^{13}C into each chamber (Högberg et al. 2010). This resulted in an overall enrichment of ∼17 atom% $^{13}CO_2$ in the chamber air and a [CO_2] of ∼500 p.p.m. directly after the release (Högberg et al. 2010). Subsequently, uptake of tracer along with dilution by respiratory release of un-labelled CO_2 from plants and

soil organisms led to a decrease down to 280–375 p.p.m. during labelling periods of 1.5–3.5 h. Despite variations in time duration of labelling in chambers ran in parallel the same day or one or a few days later, plant uptake of ^{13}C varied little, with 6.9 ± 0.7 g ^{13}C in unfertilized control plots (N = 4) and 7.0 ± 0.4 g ^{13}C in N-fertilized plots (N = 4). These small differences were the result of our decisions to adjust the duration periods of labelling individually for each chamber. Thus, we took into account the decline in photosynthetic uptake of CO_2 when the [CO_2] in the chamber air decreased (Metcalfe et al. 2017), which causes the rate of CO_2 uptake to approach the rate of plant and soil respiration simultaneously adding CO_2 to chamber air. Note that the chambers used in 2006 and 2007 were 4 m tall as compared with the 5 m tall chambers used in 2012.

Here, we tested whether it was possible to obtain a larger traceable pulse of ^{13}C by several sequential releases of tracer and by doubling the duration of the labelling period. Based on the forecast made by the Swedish Meteorological and Hydrological Institute we pre-selected a cloud-free day for the labelling. This started on the morning of 17 August 2012. We placed plastic chambers over two plots. We then released five consecutive 25 L $^{13}CO_2$ (99 atom% ^{13}C, Cambridge Isotope Laboratories, Inc, Tewksbury, MA, USA) pulses 45 min apart into the chambers. We monitored atom% $^{13}CO_2$ and [CO_2] inside the chambers using wave-length scanner cavity ring-down spectroscopy (Picarro G1101i, Picarro, Sunnyvale, California, USA). With two chambers, but only one instrument, we shifted the readings between the chambers, causing 10–20 min long gaps in the readings (Figure 1a–d).

Comparison with previous studies

For comparison with the previous study by Högberg et al. (2010) and Keel et al. (2012), we report the ratio between the maximum label in August 2012 (this study) as compared with the maximum observed in August 2007 (Högberg et al. 2010, Keel et al. 2012). The maximum value in this context was the highest mean value based on the two plots, from which we took three (soil respiration) to five (ectomycorrhizal (ECM) root tips, microbial cytoplasm C and PLFA) replicates per plot and days of sampling (at 3, 4, 7, 14 and 21 days) after labelling. We thus compare the amount of labels found in the below ground components by calculating the ratio between maximum in 2012 and maximum in 2007 using the highest mean values from the two plots labelled in August 2012 and four plots labelled in August 2007. We also extended the comparison to include the pilot study conducted in 2006 at Rosinedalsheden (Högberg et al. 2008).

Sampling and analyses

For comparison with the net ecosystem exchange (NEE) estimated at Rosinedalsheden (64°09′N, 19°05′E, 145 m above sea level) in 2007 (Metcalfe et al. 2017), we used the initial CO_2 draw-down rates at 475 p.p.m. CO_2 after each release of $^{13}CO_2$ (Figure 1a and b) to calculate a mean per plot. The NEE was calculated as described by Metcalfe et al. (2017). We also compared the soil respiratory efflux from the inner 10 m^2 in this study with data from 2007 (Högberg et al. 2010). Data on NEE and soil respiratory efflux (the sum of root and heterotrophic respiration) are important parameters to consider if plots from different locations and years are compared. If these parameters differ between studies, the comparison cannot be made with confidence.

For studies of belowground processes and biota, we used the central 10 m^2 of the labelled plots to minimize the influence of C from un-labelled trees outside the 50 m^2 plots. The ^{13}C abundance of the ECM root tips, soil respiratory efflux, microbial cytoplasm C and PLFA biomarkers for soil microorganisms were determined multiple times during the month after labelling using methods described previously (Högberg et al. 2010, Keel et al. 2012).

In brief, ECM root tips (average diameter <0.3 mm) were extracted from fresh soil samples on the day of sampling, cleaned under a dissecting microscope and freeze-dried. ECM root tips were analysed on an elemental analyser (EA) coupled to an IRMS (Europa Scientific Ltd, Crewe, UK).

Soil respiratory efflux was sampled using cylindrical 0.046 m^2 head spaces. Five gas samples sampled at 2-min intervals were analysed on a gas chromatography IRMS (Europa Scientific Ltd, Crewe, UK). The $\delta^{13}C$ value of the soil respiratory efflux was estimated using the Keeling plot method (see Högberg et al. 2008).

The $\delta^{13}C$ abundance of microbial cytoplasm was determined by the chloroform fumigation-extraction methodology (Vance et al. 1987, Högberg et al. 2008), followed by EA-IRMS analysis (Dijkstra et al. 2006) of freeze-dried soil extracts, whereas in the previous studies, the C in salt extracts were wet-oxidized to CO_2 using dichromate and then analysed by GC-IRMS.

PLFAs were extracted and analysed at James Hutton Limited (Aberdeen, Scotland, UK) following the methods of 'Bligh and Dyer' single phase chloroform:methanol:water extraction system as modified by White et al. (1979). The $\delta^{13}C$ values of PLFAs were analysed on a compound-specific IRMS (Paterson et al. 2007).

Taking tree root distribution into account

The relative contribution of roots from un-labelled trees outside the chamber to soil processes and biota inside the labelled plot was calculated based on (i) the radius of the chamber (assuming an approximately circular chamber) and (ii) the relative distribution of root C input as a function of distance from the stem (Figure 2a and b). Based on observations in nearby pine forest stands (Göttlicher et al. 2008, Högberg et al. 2008, Henriksson et al. 2021), we assumed that the relative root biomass density ($D(r)$, m^{-2}) decreases with distance from the stem (r) up to a maximum distance r_t (root length) according to:

$$D(r, r_t) = 1 - \frac{1}{\ln(2)} \ln\left(1 + \frac{r}{r_t}\right) \quad (1)$$

Because of the radial symmetry of the chamber and the root spatial distribution, we only needed to consider a location in the chamber in terms of its distance from the centre. Thus, we used polar coordinates to describe the geometry of the chamber, the tree roots and their distribution (Figure 2a and b). At each focal point x, roots come from all angles, a, and from all distances r, where $r < r_t$. For $r < y$, roots come from both inside and outside trees, whereas for $r > y$, roots come only from outside trees, which only happens for $a < a_s$ (Figure 2a). Thus, we need to determine y and a_s as follows:

$$z^2 + q^2 = r_c^2 \quad (2)$$

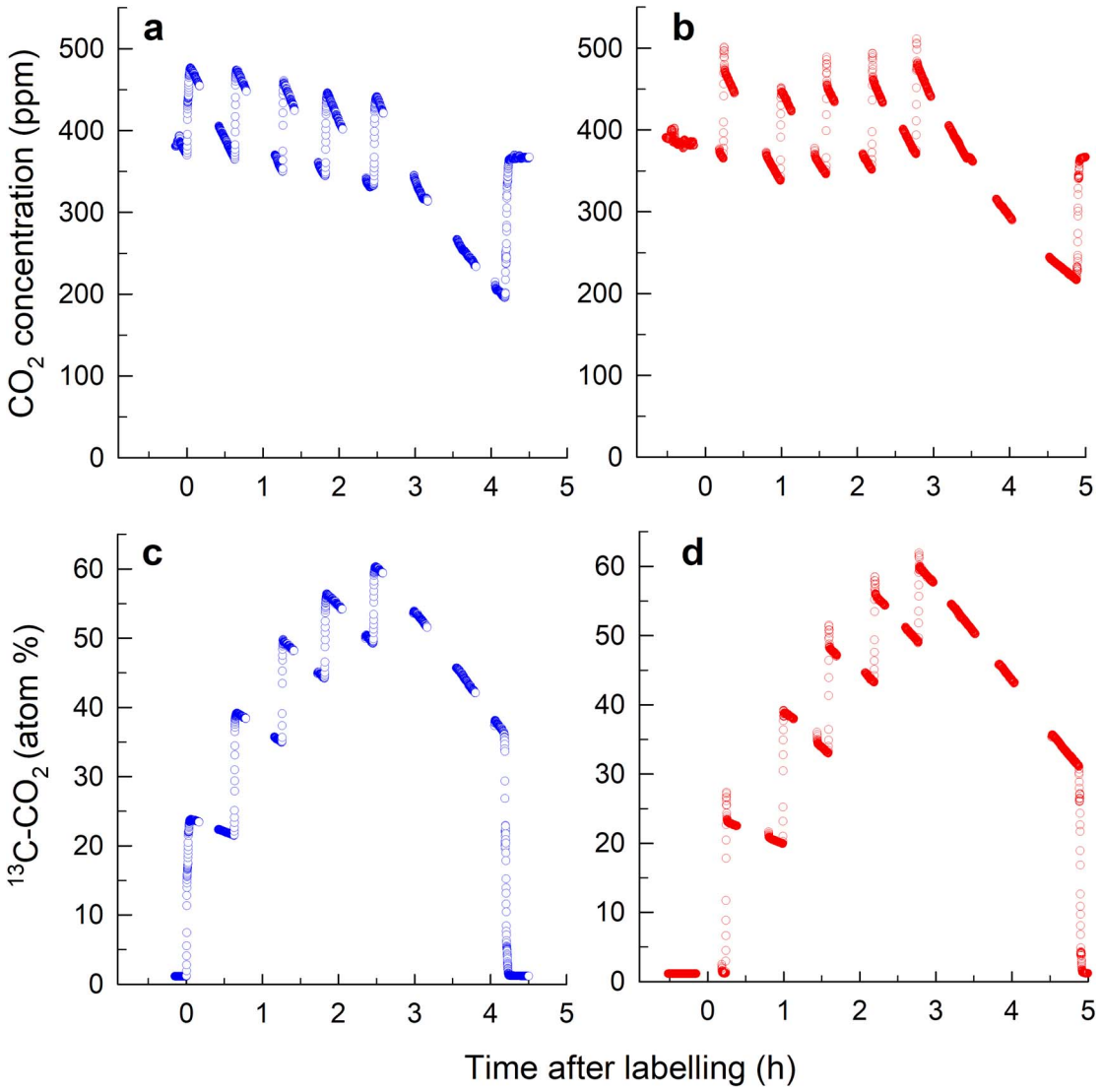

Figure 1. Time course of CO_2 concentration (parts per million) and atom% ^{13}C in CO_2 during labelling of a *P. sylvestris* L. stand. Concentration of CO_2 (a) in the control plot and (b) in the plot with added nitrogen. Atom% ^{13}C in CO_2 (c) in control plot and (d) in nitrogen addition plot.

$$(x-q)^2 + z^2 = y^2 \tag{3}$$

$$\frac{x-q}{y} = \cos a \tag{4}$$

Based on the above equations we get:

$$(\mathrm{y}\ \cos(a))^2 + {r_c}^2 - (x - \mathrm{y}\ \cos(a))^2 = y^2 \tag{5}$$

which is solved for y. Because y cannot be larger than r_t we get:

$$y\,(x, a, r_t, r_c) = \min\left(\mathrm{x}\ \cos(a) + \sqrt{\left(x^2\ \left(\cos(a)^2 - 1\right) + {r_c}^2\right)}, r_t\right) \tag{6}$$

Because $y = r_t$ when $a = a_s$ we get

$$r_t = \mathrm{x}\ \cos(a_s) + \sqrt{\left(x^2\ \left(\cos(a_s)^2 - 1\right) + {r_c}^2\right)} \tag{7}$$

which is solved for a_s:

$$a_s\,(x, r_t, r_c) = \begin{matrix} \pi - acos\left(\frac{1}{2\,r_t\,x}\left({r_c}^2 - {r_t}^2 - x^2\right)\right) & for \begin{bmatrix} x > r_c - r_t & if\ r_c > r_t \\ x > r_t - r_c & if\ r_c < r_t \end{bmatrix} \\ 0 & for \begin{bmatrix} x < r_c - r_t & if\ r_c > r_t \\ x < r_t - r_c & if\ r_c < r_t \end{bmatrix} \end{matrix} \tag{8}$$

To calculate the fraction of roots coming from outside the chamber, R, at a point x in the chamber, $R(x)$, we integrated the contributions from outside the chamber and divided by the total contributions (from inside and outside) from all distances ($r < r_t$) and all angles ($0 < a < \pi$). This calculation also accounts for the proportional increase in contributing area with distance r, i.e., for a given point in the chamber, root contributions come from a circle around this point

Table 2. Increase in ^{13}C label of selected target components and soil respiratory efflux when using the labelling method reported here as compared with previous studies. The maximum average data obtained from two plots in this study are compared with those reported by Högberg et al. (2008) for data from 2006 and Högberg et al. (2010) and Keel et al. (2012) for plots labelled in 2007 plots using the maximum values after labelling, e.g., maximum in 2012 divided by maximum in 2007. *n.d.*, not determined. *n.a.*, not applicable. Data are means of subsamples from one (in 2006) to four plots.

Ecosystem below- ground component	Time after labelling (days)			$\delta^{13}C$ in excess (‰) maximum			Times increase in ^{13}C excess		
	2006	2007	2012	2006	2007	2012	2007 *vs.* 2006	2012 *vs.* 2007	2012 *vs.* 2006
ECM root tips	4–6	4	4	3	45	244	15	5.5	81
Soil respiratory efflux	3	2–3	3	17	317	1118	19	3.5	66
Microbial cytoplasm	4	4	4	2	84	168	42	2.0	84
ECM mycelial biomarker	*n.d.*	14	14	*n.d.*	27	97	*n.d.*	3.6	*n.d.*
Overall mean	*n.a.*	6	6	*n.a.*	118	407	*n.a.*	3.7	*n.a.*

and the larger the distance from the point, the larger the circle.

$$R(x, r_t, r_c) = \frac{\int_{a_s(x,r_t,r_c)}^{\pi} \int_{y(x,a,r_t,r_c)}^{r_t} r\, D(r, r_t)\, dr\, da}{\int_0^{\pi} \int_0^{r_t} r\, D(r, r_t)\, dr\, da} \quad (9)$$

When the chamber radius (r_c) is larger than the maximum root length (r_t), there is, of course, no contribution in the centre of the chamber from trees outside it: $x < r_c - r_t$ (Figure 2b).

Results and discussion

Concentrations of CO_2 and atom% $^{13}CO_2$ during labelling

During labelling, the average [CO_2] in both chambers was 367 p.p.m., although it varied from 480 p.p.m. to 205 p.p.m. (Figure 1a and b). If the final CO_2 draw-down period after the fifth release is excluded, the average [CO_2] were 408 and 404 p.p.m. in the un-fertilized and N-fertilized plots, respectively, which was close to the diurnal average concentration in ambient air of ∼400 p.p.m. in 2012. The initial variations in [CO_2] inside the chambers of ∼100 p.p.m. reflected sequential releases of $^{13}CO_2$ followed by periods of rapid net uptake (Figure 1a and b). These variations in [CO_2] compare with diurnal variations of up to 50 p.p.m. within and below the canopy of 20-m tall forest during mid-August conditions at the ICOS (International Carbon Observatory System) tower at Svartberget, 2.5 km north of the site (www.icos-sweden.se). Clouds can add short-term variations by instantly reducing the rate of photosynthesis without affecting the rate of soil respiration, hence increasing [CO_2] in and below the canopy.

Our prime objective was to obtain a high labelling of the below-ground flux of C while keeping the [CO_2] within reasonable levels. As discussed above, the average [CO_2] inside the chambers was close to the ambient outside. In contrast, had we released all 125 L of $^{13}CO_2$ instantaneously, the [CO_2] would have exceeded 1000 p.p.m., i.e., 2.5 times the ambient. It is not known if such a temporal anomaly has consequences, but it is good practice to avoid uncertainties. We consider that the experimentally induced short-term deviations from the natural dynamics of [CO_2] during 4.0–4.5 h have no relevant impact on biota and processes in the soil, in which labelled C is observed 3–4 days later and onwards (Högberg et al. 2010, Keel et al. 2012, Table 2).

In both chambers, the atom% ^{13}C of CO_2 was 23 directly after the first release of labelled CO_2 (Figure 1c and d) and declined until the subsequent release of tracer. Each additional release increased the atom% ^{13}C of CO_2 up to the maximum of 61 atom% after the fifth and final release in this experiment. The fifth release was followed by a period of draw-down towards 200 p.p.m. CO_2 to achieve high assimilation of the label. The full sequence from the first labelling to the completion of the labelling was 40 min slower in the N-fertilized chamber as compared with the control chamber, possibly because of a lower needle biomass. The average atom% ^{13}C of CO_2 was 42.1 and 41.3 atom% in the control and N-fertilized chambers, respectively. This is roughly four times higher than during the experiments conducted by Högberg et al. (2010).

For each release of 25 L of $^{13}CO_2$, the increase in atom% ^{13}C in the CO_2 in the chambers became progressively smaller (Figure 1). This is expected since respiration of un-labelled CO_2 restricts the maximum level of atom% ^{13}C of the CO_2, which can be obtained in the chambers in a pulse-labelling experiment of this kind. We fitted an exponential equation to the data (Figure 3) from the five additions in this experiment:

$$y = a\left(1 - e^{-bx}\right) \quad (10)$$

where y is atom% ^{13}C in CO_2, x is the litres of $^{13}CO_2$ added, and a and b are constants. We found a good fit ($R^2_{adj} > 0.999$) for the formula :

$$y = 68.56\ \left(1 - e^{(-0.017x)}\right)$$

Hence, a maximum of 68.56 atom% ^{13}C could be obtained in the chambers under the prevailing experimental conditions. We reached 61 atom% ^{13}C. To approach the calculated maximum would require acceptance of diminishing returns of investments in tracer.

Comparison with previous studies

For a comparison with the previous studies to be of interest, it is essential that the forests studied at Åheden and Rosinedalsheden are similar (note that the studies by Högberg et al. (2008) and Högberg et al. (2010) were conducted in the forest at Rosinedalsheden). They are both boreal *P. sylvestris* forests growing on N-poor soils and under climatic conditions of a short summer; snowmelt peaks in late April (Laudon et al. 2021). Net photosynthesis should peak in July (Troeng and

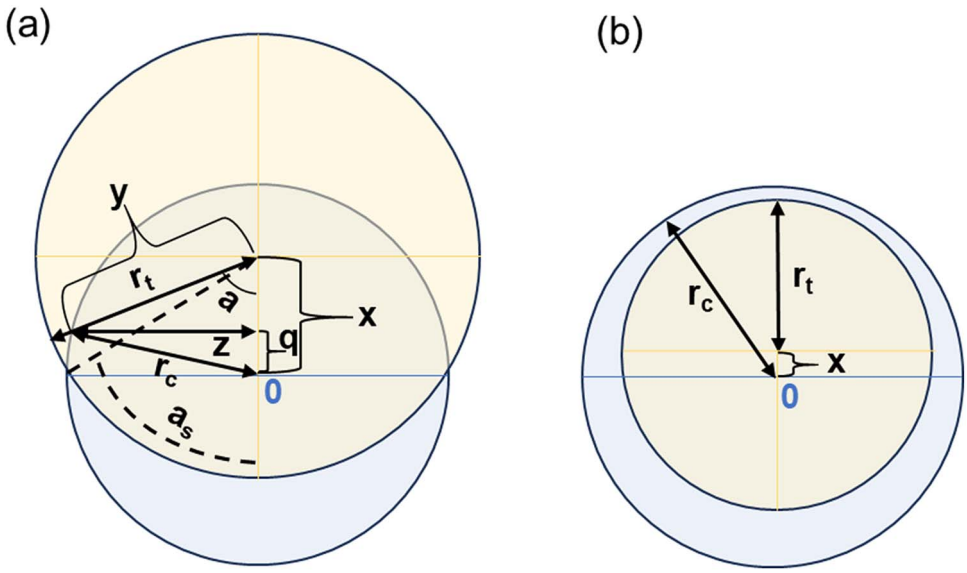

Figure 2. Geometry of *P. sylvestris* L. root distribution and a chamber represented by circles. In (a) the maximal tree root length (r_t) is larger than the radius of the chamber (r_c). The relative influence of outside trees increases with distance (x) from the chamber centre (0). At each x, roots come from all angles, *a,* and the contributions from outside and inside the chamber vary with *a*. For $a = a_s$ the two circles intersect (dashed line) and for $a < a_s$ there is no contribution from outside trees. In (b) the chamber radius (r_c) is larger than the maximal root length r_t, which means that in the centre of the chamber (for $x < r_c - r_t$) there is no impact of outside roots.

Linder 1982), and a major increase in belowground C allocation occurs in late summer (Högberg et al. 2010). We note that the age of the trees, soil characteristics like pH, C/N ratio and respiration are similar, and especially that the ecosystem NEE are similar (Table 1). Metcalfe et al. (2017) reported that NEE at mid day in the summer of 2007 in the eight plots (four N-fertilized, four unfertilized) studied by Högberg et al. (2010) at Rosinedalsheden was 1.08 ± 0.06 CO_2 m^{-2} h^{-1} at ~475 p.p.m. CO_2. Our estimate for the two plots studied here was 1.16 ± 0.05 g CO_2 m^{-2} h^{-1}, i.e., close to the mean observed in the previous study. Further, the rates of soil respiration (which is the sum of heterotrophic respiration and tree belowground respiration) were 0.39 ± 0.02 g CO_2 m^{-2} h^{-1} at Rosinedalsheden as compared with 0.42 ± 0.03 at Åheden. This means that the CO_2 exchange differed little between the two sites. Hence, we considered it appropriate to compare the studies despite the distance of 7.2 km between them.

Thus, we compare (Table 2) the labelling of three belowground components and of the soil respiratory efflux as obtained in this study with those in the previous full-scale study (Högberg et al. 2010, Keel et al. 2012) and in the pilot study (Högberg et al. 2008). We note that it took roughly the same time before the maximum labelling of belowground components occurred in the studies, with a range from 3 to 14 days, depending on the object of study (Table 2). Here, we mainly focus on comparing our results with those from the previous full-scale study. Using the high tracer labelling approach, the labelling was 5.5 times higher in ECM roots, 2.0 times higher in microbial cytoplasm, 3.6 times higher in the PLFA Biomarker 18:2ω6,9 for ECM fungal mycelium, and 3.5 times higher in the soil respiratory efflux. These figures compare with the expected based on the four fold higher $^{13}CO_2$ concentration during labelling. In Table 2, we also make a comparison with the results of the very low tracer addition, 5 L $^{13}CO_2$, used in a pilot study at Rosinedalsheden in August 2006 (Högberg et al. 2008). This reveals the major differences in labelling of below-ground components after adding 5, 25

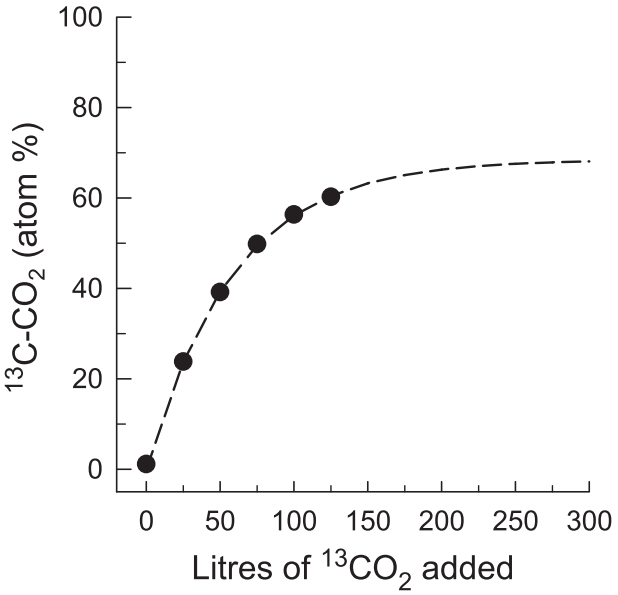

Figure 3. The relation between litres of $^{13}CO_2$ added and the atom% ^{13}C of the CO_2 in the air in the control chamber. Filled circles show data from the five additions of tracer. The broken line shows the relation as predicted by the general Eq. (10).

or 125 L of $^{13}CO_2$. The results also reflect that the chambers were only 4 m tall in 2006 and 2007 as compared with 5 m tall here, which caused the concentrations of tracer to deviate from the relations 1:5:25 expected if the chambers were of equal volume. Taking the differences in chamber volume into account, the expected relations are 1:5:20, i.e., a four fold higher labelling in 2012 as compared with in 2007.

Frequent sequential releases of $^{13}CO_2$ result in a broader peak of labelling in target organisms and processes as compared with a single release of tracer. This affects calculations of turnover rates in above-ground components like needles

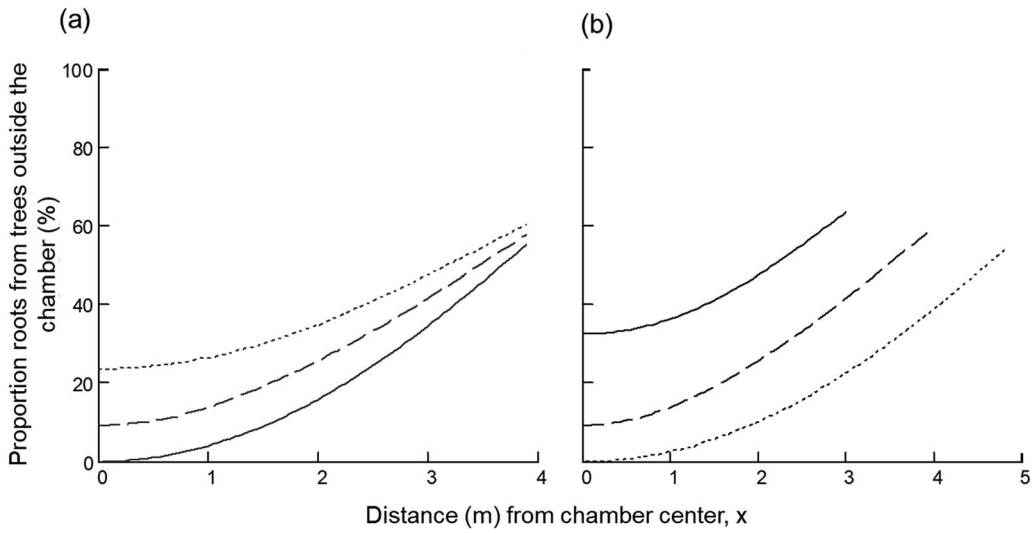

Figure 4. The proportion of un-labelled *P. sylvestris* L. roots coming from trees outside the chamber out of all tree roots inside the chamber, *R* (in %), as (a) a function of maximum root lengths, r_t, showing maximum root lengths of 3 m (solid line), 4 m (dashed line) and 5 m (dotted line) and in (b) as a function of distance from chamber centre, *x*, for root length 5 m and different chamber radius, r_c, at 3 m (solid line), 4 m (dashed line) and 5 m radius (dotted line). Sampling of below-ground components was done within 1.78 m distance from the chamber centre.

and phloem sap, in particular, but less in below-ground components, where the tracer reaches a maximum of 3–14 days after labelling (Table 2).

Taking the influence of un-labelled roots into account

The size of chambers, especially the width, is of crucial importance for studies that aim to reflect ecosystem-scale processes, in particular when the labelling of above- and below-ground components are described in detail. The fact that tree roots commonly extend much further from their stems than do the branches (Stone and Kalisz 1991) is a complication. In nearby *P. sylvestris* forest stands, tree roots reached ~5 m from tree stems (Göttlicher et al. 2008, Högberg et al. 2008, Henriksson et al. 2021), such that any circular area of 1 m^2 was occupied by the roots of ~10 trees (Göttlicher et al. 2008, Henriksson et al. 2021). Gao et al. (2021) also reported that most of the labelled root activity was within 4–5 m from tree stems in a temperate pine forest. With 730 stems ha^{-1} in their study, the calculated average distance between stems should be 4 m, which means that root systems must have overlapped.

We used octagonal chambers covering 50 m^2, in which we sampled soils, roots and soil biota in the central 10 m^2. Thus, we conclude that roots of un-labelled trees outside the chambers (Högberg et al. 2008) had an influence on the area studied. In the following discussion, we assume that the biomass of active roots of trees from outside the chamber decreases towards the centre of labelled plots in the way shown by ^{15}N tracer in Högberg et al. (2008) and Göttlicher et al. (2008). We also assume that the central 10 m^2, from which we took samples of tree roots and other soil biota, is circular. Based on these assumptions, we estimate in Eqs. (1–9) that ~15% of the soil biota and associated soil processes in the central 10 m^2 is affected by C from un-labelled trees outside the chamber (Figures 2 and 4). Hence, while aboveground parts of trees within the 50 m^2 of the chamber were all labelled, roots and other biota sampled by soil coring in the central 10 m^2 would be 85% labelled. Just inside the margin of labelled plots, 60% of the C would come from roots of un-labelled trees and only 40% of the C in below-ground biota and processes would come from labelled trees (Figure 4a).

It is an important observation that reducing the area of the patch with labelled trees results in an increase in the contributions of un-labelled trees to soil biota and processes (Figure 4b). A physical barrier, trenching, would hinder this, but would introduce an undesired input of un-labelled C from dying roots and root-associated organisms and would also affect the trees inside by severing roots extending outside the chamber. A barrier would also disturb below-ground interactions among trees.

The presence of un-labelled roots from trees outside the chamber confounds attempts to match the above-ground and below-ground C budgets. One can reduce the problem of the impact of roots of un-labelled trees by increasing the area covered by chambers. However, adding 1 m of radius to the 4 m adds 56% to the volume of chambers, and increasing the height of 5 m by 1 m adds 20% to the volume of chambers increasing the quantity of labels needed accordingly. Furthermore, a larger volume of chambers demands more energy for cooling. At this remote location, we used a mobile diesel-driven engine with a capacity to produce 35 kW. Cooling of the two chambers required ~25 kW under full sunlight at midday (25 °C), i.e., 0.05 kW per m^3 of chamber air.

Concluding remarks

With our approach of pulsed tracer release, we achieved a significantly higher ^{13}C labelling of different below-ground compartments compared with single pulse labelling while keeping [CO_2] at reasonably low levels. Since higher labelling was found in all compartments investigated, we assume that this should be the case for other compounds and organism groups as well. Recent developments in molecular biology have opened up new opportunities to identify soil organisms and to study gene expressions (e.g., Law et al. 2022). If such techniques are combined with stable isotope probing (SIP), it becomes possible to couple the taxonomic specificity

of molecular biomarkers (e.g., PLFAs, DNA and RNA) to quantitative measures of ecosystem processes based on SIP. This step requires a high level of labelling, which, until now, has been possible under laboratory conditions or by using small plants in the field.

However, field-scale labelling with trees is desirable from many points of view. Such experiments involve the soil microbial community of interest unaltered by experimental installations and can encompass seasonal variations in tree belowground C flux. Thus, results are directly translational to the ecosystem level. As we show here, multiple-release of labelled CO_2 is a useful method to achieve high labelling of soil processes and organisms under natural conditions in the field. We also heightened the need to consider the role of un-labelled C from trees outside the chamber. Further improvements are possible (e.g., by increasing the labelled area), but the wish to maintain an undisturbed system, natural levels of [CO_2] and a short pulse of tracer puts limits to the level of tracer that can be obtained in target organisms, compounds and processes.

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Authors' contributions

P.H. (Conceptualization, Funding acquisition, Investigation, Resources, supervision, Writing—original draft, Writing—review & editing), C.K. (Data curation, Investigation, Formal analysis, Writing—review & editing), O.F. (Formal analysis, Methodology, Visualization, Writing—review & editing), N.H. (Investigation, Writing—review & editing), H.L. (Investigation, Writing—review & editing), E.I. (Investigation, Writing—review & editing), V.H. (Investigation, Writing—review & editing), T.N. (Funding acquisition, Investigation, Writing—review & editing) and M.N.H. (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing—review & editing). Author contribution statements follow Contributor Roles taxonomy; https://credit.niso.org/.

Conflict of interest

None declared.

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Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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