2. Carbon and nutrient cycling

S Vicca (editor)

The **cycles of carbon and nutrients (Chapter 2)** involve many ecological processes that are all potentially sensitive to environmental change (Bai et al., 2013; Yue et al., 2017). Key terrestrial ecosystem processes include photosynthesis, aboveground and belowground plant growth, autotrophic and heterotrophic respiration, organic matter decomposition and nutrient mineralisation. Even minor changes in any of these processes, or in the balance between them, can have implications for ecosystems as well as individual organisms. Ecosystem responses to environmental changes can affect important services that terrestrial ecosystems provide to humanity, such as provisioning of food and fibre, water regulation, and carbon sequestration (Trumbore et al., 2015). Therefore, the assessment of responses of biogeochemical processes to a changing climate in global-change studies is important from a fundamental and applied perspective.

In the carbon and nutrient cycling chapter, we focus on the main components of the terrestrial carbon cycle, including aboveground and belowground processes, pools, and fluxes. Nutrients are also included, but only those pools and processes that are linked to carbon cycling and ecosystem feedbacks to the climate. We distinguish between plant and soil measurements. We also provide three thematic protocols that are particularly relevant when considering carbon and nutrient cycling processes within terrestrial ecosystems (plants, soil, ecosystem).

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How to cite a protocol:

E.g. To measure CO_2 efflux, we use the method described in protocol 2.1.4 Plant respiration in the Supporting Information S2 Carbon and nutrient cycling in Halbritter et al. (2020).

Halbritter et al. (2020) The handbook for standardised field and laboratory measurements in terrestrial climate-change experiments and observational studies (ClimEx). *Methods in Ecology and Evolution*, *11*(Issue) pages.

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Halbritter et al. (2020) The handbook for standardised field and laboratory measurements in terrestrial climate-change experiments and observational studies (ClimEx). *Methods in Ecology and Evolution*, *11*(Issue) pages.

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2.1 Plants

Plants convert light energy and atmospheric carbon dioxide (CO_2) into plant biomass via photosynthesis and respire it back to the atomsphere. Plant biomass serves as the energy source for all other trophic levels in the ecosystem. Aboveground and belowground plant biomass feeds the world population.

2.1.1 Aboveground plant biomass

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Measurement unit: g dry biomass m^{-2}; Measurement scale: plot; Equipment costs: €-€€; Running costs: none; Installation effort: low to medium; Maintenance effort: low; Knowledge need: low to medium; Measurement mode: manual

Aboveground plant biomass (AGB) is the total amount of plant-derived living and dead organic matter. Total AGB can be used to estimate total aboveground carbon (C) and nutrient stocks. Aboveground net primary production, ANPP (g m^{-2} yr^{-1}) can be estimated from time series measurements of AGB for a given growth period (Clark et al., 2001; see protocols in Scurlock et al., 2002 and Ruppert & Linstädter 2014). ANPP is a key ecosystem characteristic and of fundamental importance for many aspects of matter and energy fluxes in terrestrial ecosystems (Cleveland et al., 2011). At the same time, it is one of the best-documented estimates of key ecosystem services such as forage production (Lauenroth & Sala 1992; Ruppert et al., 2015). Species-specific AGB is also an important metric of plant fitness, particularly for perennial species (Younginger et al., 2017). AGB is often used as a rough indicator of belowground biomass (BGB) that can only be measured destructively. A fixed belowground:aboveground ratio is typically applied to estimate BGB from AGB (see review in Addo-Danso et al., 2016) although a meta-analysis of global data shows that results have to be interpreted with care (also see protocol 2.1.2 Belowground plant biomass, Mokany et al., 2006).

In this protocol we introduce measures of AGB only and direct the reader to literature on ANPP estimates at the end of this section.

Green AGB fixes atmospheric CO_2 and provides an estimate of the carbon sequestration potential of an ecosystem. The allocation of atmospheric carbon to the soil by plants drives soil processes by enhancing plant-microbial interactions (e.g. Kuzyakov & Domanski, 2000), and is the basis for plant-mycorrhizal interactions (Grayston et al., 1997). The assessment of AGB provides important information on ecosystem-level carbon and nutrient cycling and is used in greenhouse gas inventories. Accurate field-based measurements of AGB across ecosystems are urgently needed to improve current regional- to global-scale vegetation models that are, amongst others, driven by AGB (Scheiter et al., 2013; Martin et al., 2014). Changes in AGB are important to assess during, for example, experimental climate manipulations (e.g. Kongstad et al., 2012; Tielbörger et al., 2014). Our assembley of protocols includes AGB assessments using destructive and non-destructive methods useful for climate-change studies and

we describe appropriate methods for the ecosystem of interest (i.e. grassland, shrubland, forest). The ecosystem type primarily defines the appropriate method (e.g. Traxler, 1997; Smart et al., 2017, Supporting documentation). The assembled protocols can also be used to study AGB in other types of studies such as global-change experiments (land-use change, nutrient additions, etc.) and environmental gradient studies.

2.1.1.1 What and how to measure?

Defining and marking AGB observation plots

Methods that assess AGB depend on the ecosystem type, such as grassland, shrubland, or forest. Measurements on AGB are collected from representative and equally sized observation plots per vegetation type (Singh et al., 1975; Fahey & Knapp 2007). It is important that the AGB observation plots are managed the same way as the surroundings. For example, cutting, grazing, or fertilising should be carried out as usual. AGB observation plots can be located within permanent experimental plots or along transects.

Plot size depends on plant community composition and plant size, and varies systematically across vegetation types (Smart et al., 2017, Supporting documentation). An appropriate plot size may be determined using a nested plot design (Figure 2.1.1.1 a) or a multi-plot approach (Figure 2.1.1.1 b). The number of plant species is determined in plots of doubling sizes (1 to 4 doubling steps, or more). The suggested minimum plot size is when the percentage increase in plant species is lower than 10% when the plot size is doubled. Often, this method results in plot sizes that are impractical to sample (because they are too large); the plot size may then be reduced to reflect 80% of the species, or 90% of the most productive species present. Commonly used dimensions for AGB observation plots are listed in Table 2.1.1.1. The number of replicated plots should reflect the observed heterogeneity in plant composition in the studied ecosystem. The more homogeneous the area is, the smaller the AGB observation plots can be (Traxler, 1997).

In forests, plot size depends on tree size. Variable radius plots are often used, where the plot size increases as a function of tree diameter, increasing the sample size for the large trees that most influence the biomass (Monserud & Marshall, 1999).

Table 2.1.1.1 General guidelines for the size of AGB observation plots.

Ecosystem	Dimension of area (m*m)	References
Temperate forest – tree layer	10 x 10 to 50 x 50	Traxler, 1997
Tropical forest and savanna – tree layer	20 x 20 to 100 x 100	Alder & Synnot, 1992
Forest – understorey	subplots of 1 x 1 to 2 x 2	Traxler, 1997
Shrubland	4 x 4 to 10 x 10	Traxler, 1997

Heathland	2 x 2 to 4 x 4	Traxler, 1997
Moor	0.1 x 0.1 to 1 x 1	Traxler, 1997
Grassland, savannah and alpine/arctic vegetation – grass layer	0.25 x 0.25 to 1 x 1	Bertora et al., 2012; Linstädter & Ruppert, 2015

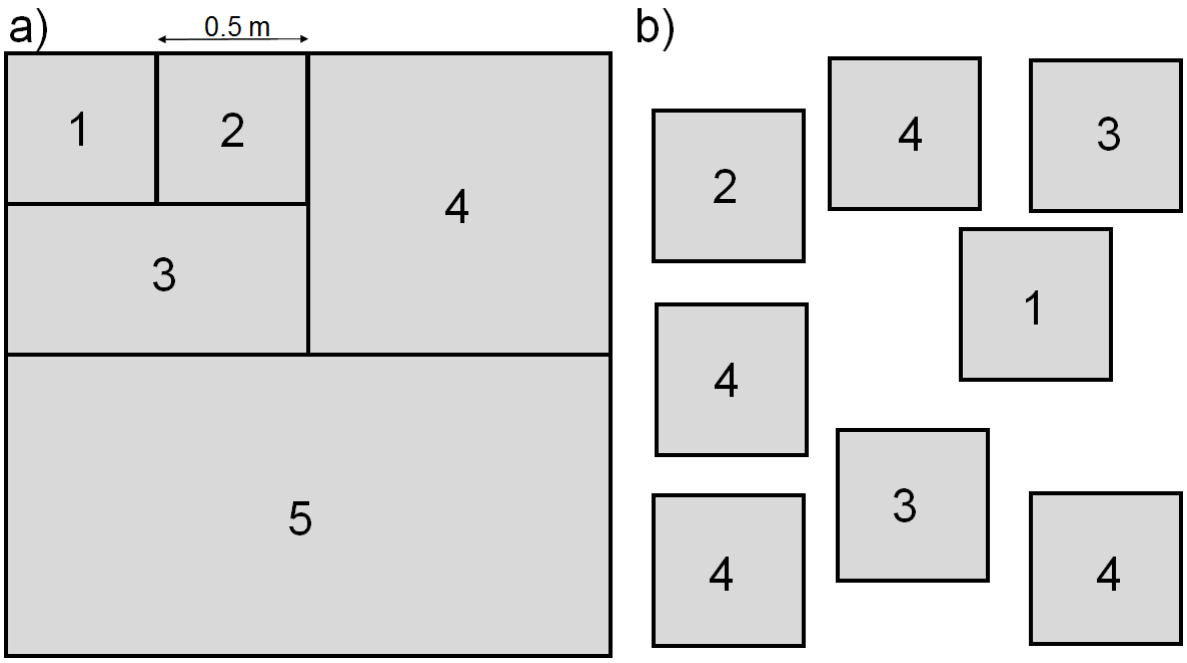

Figure 2.1.1.1 Identification of minimum plot size for AGB observation plots. The number of plant species is determined in plots of doubling sizes, either (a) with a nested plot design (here with doubling steps 1–5) or (b) with a multi-plot approach (here with doubling steps 1–4). The minimum plot size is the plot where the percentage increase in plant species is lower than 10%. Graphics adapted from Mueller-Dombois & Ellenberg, 1974 and Beierkuhnlein, 2006.

AGB observation plots should be permanent and marked. Aluminium tubes or other metal tags at each corner are recommended. In study sites where visible plot markers may be stolen or removed (such as experimental sites in the Global South where the market price of aluminium or other metals may provoke theft), we recommend a combination of aboveground with belowground tags. For aboveground tags, materials with a low market price such as plastic sticks are most feasible. For belowground tags, we recommend magnets (buried to a depth of 15–25 cm); these are retrievable with a magnet detector. If the area is subject to unpredictable soil compaction (e.g. through frost or heavy machines), retrieval of magnets can be very difficult. For larger experiments, a GPS reading of plot corners, preferably with a differential GPS, is an alternative. Marking plots may be problematic in, for example, grazed ecosystems where permanent structures may increase the concentration of grazing animals and lead to preferential

grazing within plots. In this case, Dodd (2011) may be consulted for alternative plot-relocating methods (see *Special cases* below).

Measuring AGB

Destructive harvesting is generally a more accurate estimate of AGB than a non-destructive AGB assessment. However, repeated non-destructive estimates of AGB are preferred in manipulation experiments (e.g. Kongstad et al., 2012; Tielbörger et al., 2014) if cutting or grazing is not part of the disturbance regime of the studied ecosystem. Non-destructive proxies of AGB can normally be measured with a little training, but measurements are time-consuming. Field spectroscopy requires more elaborate (technical) skills, and can be hampered by difficult external requirements such as unfavourable weather/cloud conditions in tropical environments (Ferner et al., 2015).

The methods described below are divided into 1) non-forest AGB methods and 2) forest AGB methods. Shrublands may fall into either of these two categories. In **non-forest** ecosystems (low-vegetation such as agriculture, grasslands, shrublands, etc.), the most frequently used non-destructive AGB methods are 1) point-intercept method (Goodall, 1952; Jonasson, 1988; Damgaard et al., 2009; Hudson & Henty et al., 2009; Valolahti et al., 2015), 2) field spectroscopy (Pearson et al., 1976; Milton et al., 2009; Ferner et al., 2015), and 3) visual cover estimation (Braun-Blanquet, 1932; Sykes et al., 1983; Peet et al., 1998). These methods require a destructive harvest outside the experimental area to obtain AGB estimates from the non-destructive vegetation assessments. Comparisons of methods show that each of the above methods can be effective in estimating AGB (Onodi et al., 2017a).

Measuring AGB in agriculture, grasslands, and shrublands (non-forests) (Gold standard)

a) Destructive harvesting (Bertora et al., 2012). AGB that is rooted within the AGB observation plots is harvested by clipping the vegetation homogeneously above (e.g. 2 cm) the soil surface except if the vegetation includes substantial amounts of rosette-plants or includes aboveground stolens. Slow-growing, small vegetation (e.g. in alpine/arctic ecosystems) may be cut homogeneously closer to the soil surface (e.g. 0.5 cm). For shrubs rooted within the observation plots, leaves and the current year's woody growth should be collected. If woody vegetation is part of the plot, a mixture between the gold and bronze standard can be used to assess AGB of grass vegetation (destructive harvest) and shurbs (non-destructive harvest, see below). The timing of sampling depends on the vegetation type and land-use practice. For further details see *Timing of sampling* below. Destructive harvesting is suggested for:

- agricultural and managed ecosystems where the harvest is part of the management practice
- herbaceous-dominated ecosystems (NutNet protocol): AGB that is rooted in parts of the observation plots may be harvested destructively over time (NutNet protocol). For example, if AGB is monitored over four years, 25% of the AGB observation plot can be harvested each year
- manipulation plots that simulate grazing (Linstädter & Ruppert, 2015) the end of an experiment.
- Harvested AGB is then sorted into live and dead biomass, growth form, species, and plant organs when the plant material is still wet (Table 2.1.1.2). After sorting, plant material is dried at

65 °C to constant weight. AGB is calculated by summing up all the dry-weight fractions and is expressed in grams of dry biomass per m^2.

Table 2.1.1.2 Hierarchy of detail documented for plant material from destructive harvests. (Fresh) Plant material should, at the minimum, be divided into living, dead, and morbid structures of the different plant functional types. Species information is good to collect. Ideal is information on plant organs (Bertora et al., 2012; INCREASE).

Recommended		**Good to have**	**Nice to have**
Alive	Bryophytes/lichens	Species	location (soil surface/stem)
	Grasses		grasses/sedges/rushes
	Forbs		leaves/flowers
	Woody (shrubs)		stem/leaves/flowers
	Woody (trees)		current year's woody growth
Dead	Grasses	Species	leaves/flowers
	Forbs		stem/leaves/flowers
	Woody		current year's woody growth

b) Non-destructive point-intercept method (Goodall, 1952; Jonasson, 1988; also see 4.8 Plant community composition). The point-intercept method provides detailed information about plant community composition, 3-D canopy structure, reproduction, survival, mortality, community development, and plant competition (Jonasson, 1988; Damgaard et al., 2009; Kongstad et al., 2012). The assessment of dead and living plant parts can be used to estimate litter production of grassland species where litter remains attached to the plants ('standing dead matter').

To use the point-intercept method, the AGB observation plot is divided into a reference grid by using a mountable frame above the plant canopy (Picture 2.1.1.2). The four corners of the frame need to be permanently marked. At each grid point, a pin is lowered vertically through the vegetation. For each plant hit along the pin, height and plant species are recorded (field notes or dictaphone). The hits of all species and plant organs (flower, annual shoot), status (alive, dead), and height are documented. Measurement protocols are detailed in the INCREASE protocol (INCREASE, 2014). It is also common to do a 2-D recording without height of pin hits on species. It is very fast but provides only plant cover estimates and is not suitable for converting pin-hits to biomass (see *AGB conversion to Aboveground Net Primary Production* below).

To convert pin-hits surveyed in the AGB observation plots to actual AGB, vegetation plots of the same size and the same vegetation composition have to be surveyed outside the experimental area. After surveying these reference plots, AGB (and optionally BGB) is harvested and sorted, and dry AGB is determined (see *Destructive harvest* above). We suggest surveying a minimum of ten representative vegetation plots outside the experimental area. If the vegetation at your site is heterogeneous, the calibration should be performed for different vegetation compositions. Reference surveys outside the experimental or observation plots need to be repeated with time when a change in vegetation has occurred, for example after pest outbreaks and vegetation responses to experimental treatments (Onodi et al., 2017b).

For each survey plot, the total number of hits for each species on the pin is expressed as a proportion of all pin-hits in a plot. The proportions of species pin-hits are then correlated with the determined dry plant biomass (Figure 2.1.1.2, INCREASE, 2014). The derived relationship for each species is used to convert pin-hits measured in the AGB observation plots to biomass per area. The total AGB in each AGB observation plot equals the sum of biomass of all species; AGB needs to be expressed per m^2. If the vegetation surveys are carried out at the same time of year for consecutive years, these measures of AGB can be used to estimate ANPP (see section *Special cases* below).

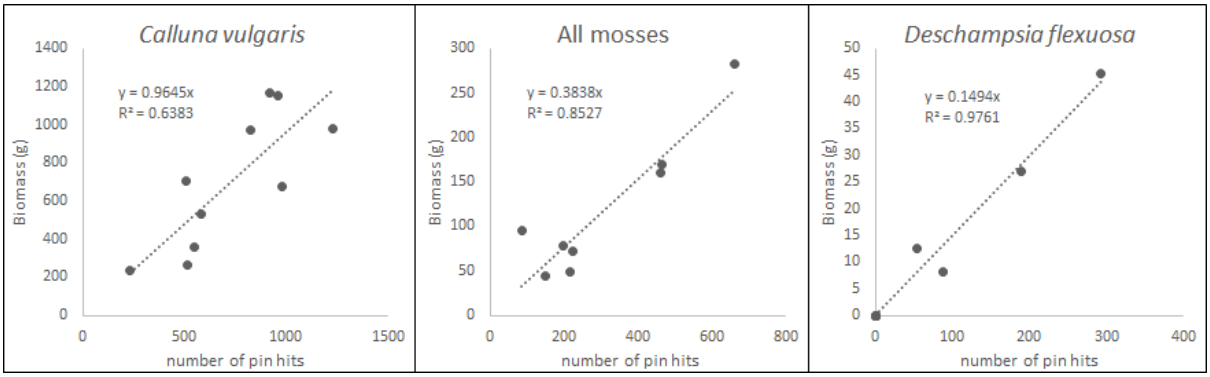

Figure 2.1.1.2 Correlation between pin-hits and harvested biomass in areas outside the AGB observation plots ((data from Clocaenog field experiment, UK; Domínguez et al., 2015). Number of pin-hits of each species is correlated with the measured (dried) biomass for each plot of a defined area. A relationship of the form y = mx + n where y is the measured dry biomass, x = pin-hits, m = derived relationship for each species, and the intercept n = zero given the assumption that there are no pin-hits, the species is not present (thus the biomass = 0). The more information derived from the point-intercept survey (e.g. green leaves, dead leaves, stems of each species), the more accurate the relationships will be.

Picture 2.1.1.2 Point-intercept survey in climate change experimental plots. The AGB observation plot is located within an experimental plot; the corners of the AGB observation plot are marked with four sticks (top left). The frame (top right) can then be put onto the sticks. The surveyor is positioned on a plank resting on the experimental structure avoiding disturbance of the vegetation and soil (bottom left). A frame with 10 cm distance points is used as guidance for the survey-pin that is lowered into the vegetation (bottom left and right). Pin-hits are recorded using a dictaphone. Notes with vegetation codes are used for easier decoding afterwards. Photos: Inger Kappel Schmidt (top left to bottom left) and Alwyn Sowerby (bottom right).

Measuring AGB in agriculture, grasslands, and shrublands (non-forests) (Bronze standards)

The ''Normalized Differential Vegetation Index'' (NDVI) and visual estimation cover estimates are both proxies of AGB and need to be converted to AGB values by using measurements of destructive harvests outside the experimental area as described for the gold standard.

a) NDVI (Pearson et al., 1976; Milton et al., 2009). The most frequently used proxy for AGB is the NDVI (Tucker, 1979) which correlates with green AGB (Tucker & Sellers, 1986; Ferner et al., 2015). It is the quickest method to assess the living green biomass (Onodi et al., 2017b) and is a good measure to track seasonal changes within the green plant biomass (Gamon et al., 1995). Using a portable multi-spectral radiometer, incoming and reflected light intensity is measured at different wavebands in each AGB observation plot. The weather conditions need to be recorded as precisely as possible and date and time are crucial information. Uniform overcast without direct radiation is the preferred condition during measurements (Bertora et al., 2012), although newer equipment is able to account for changes in light intensity. We advise reading the manual of your equipment carefully.

An NDVI sensor can be hand-held or mounted on a boom above the AGB observation plots. The sensor needs to be placed higher above the canopy the larger the AGB observation plot is to capture the entire biomass within the plot. For example, Onodi et al. (2017b) levelled an NDVI sensor 1.8 m above a 0.25 m^2 plot and 2.8 m above a 1 m^2 plot. The use of a boom reduces the interference of the operator with the measurements. Multispectral radiometers are usually composed of paired sensors for NIR_{810} and R_{660} and both wavebands can be measured simultaneously (INCREASE, 2014). Using a spectrometer with sensors for both incoming and reflected light provides a more robust result and is thus not dependent on a clear sky (INCREASE, 2014).

NDVI is calculated as $NDVI = (NIR_{810} - R_{660}) / (NIR_{810} + R_{660})$, where NIR_{810} is the reflectance measured at the near-infrared (NIR) waveband (centred at 810 nm) and R_{660} is the reflectance measured at the red (R) waveband (660 nm). Note that NDVI goes to saturation at high canopy density and thus is less effective when vegetation becomes dense (leaf area index (LAI) above 2) (Gamon et al., 1995).

b) Visual cover estimation (Braun-Blanquet, 1932; Sykes et al., 1983; Peet et al., 1998). It has been historically used to assess the plant community composition of an ecosystem (see protocol 4.8 Plant community composition). Visual cover estimation can also be used to estimate AGB in climate-change experiments (Zhang & Welker, 1996; Tielbörger et al., 2014). Percentage cover is typically estimated as 25%, 30%, 35% etc. above 20% cover, full numbers between 2% and 20%, and to one decimal digit when cover is below 2%, i.e. 1.5% or 0.4% (Ónodi et al., 2017a). In case of multiple observers, training may be needed to facilitate consistency (Sykes et al., 1983). Based on calibration in plots with destructive sampling, the estimated cover can be converted to AGB. It has been shown that visual cover estimation can be similar in accuracy to field spectroscopy and the point-intercept method in estimating AGB (Ónodi et al., 2017a, 2017b).

Measuring AGB in forests (Gold standard)

In forests, AGB can be estimated using allometric relationships, which use simple measures of diameter, and sometimes other variables, to estimate more complex variables, such as leaf biomass. The commonly used model is a power form equation ($A = \alpha B^x + \ldots + \beta Z^y$), with coefficients derived from empirical measurements. Often the equation form is simplified to $A = aD^x$, where D is the tree diameter. Because allometric equations can vary considerably (Ketterings et al., 2001), the gold standard uses site-specific allometric equations for each species.

Several alternatives have been suggested to improve precision and generality. Chave et al. (2014) show that at least three tree parameters are needed to calculate robust, general allometric equations: 1) maximum tree height, 2) diameter at breast height (DBH), and 3) wood specific gravity. Monserud & Marshall (1999) also accounted for height to the base of the crown and competition indices, which account for differences in tree shape with variable stand density. Ketterings et al. (2001) use a function of tree height and wood density to parameterise across a range of sites, generalising their equations such that site-specific equations were no longer necessary.

To drive allometric equations, the following predictor variables are often required:

1. Maximum tree height is measured through trigonometric methods using laser equipment or mechanically with a telescopic stick with decimetre marks (Pérez-Harguindeguy et al., 2013).
2. DBH is defined as diameter at a height of 1.3 m. It is best measured using a laser relascope, callipers, or a DBH-tape. In case of buttresses or forked stems occurring above or below 1.3 m, DBH is measured just above the buttresses or below the fork. (Hoover, 2008; Avery & Burkhart, 2015).

   Dendrometers are used to measure changes in stem size in a single radius, across the diameter or around the circumference of a stem at a particular height. For within-season measurements of changes in stem diameter or radius, both manual and automatic methods are available (Drew & Downes, 2009). Stainless steel band dendrometers, installed at breast height, can be used to determine the onset and cessation of diameter growth, as well as the seasonal variations in growth. The measurements can be made manually using digital callipers, but there are also band dendrometers with automatic recording. Point dendrometers can measure the changes in stem, branch, or coarse root radii with a resolution of less than 1 µm. At such high resolution, diurnal swelling and shrinking of the stem can be seen (Zweifel & Häsler, 2001). These stem radius changes consist of actual radial growth and shrinkage and swelling from the inflow and outflow of water (cf. Zweifel et al., 2006).
3. Wood specific gravity is measured as oven dry biomass divided by fresh volume. Biomass for this measurement is obtained by taking a wood core at breast height using an increment corer, aiming for the pith of the tree. Fresh volume is calculated from the core cylinder with length measured immediately after sampling the core and removing the bark and diameter defined by the increment corer (Williamson & Wiemann, 2010; Pérez-Harguindeguy et al., 2013). Data can also be taken from databases such as TRY (Kattge et al., 2011), DRYAD (Zanne et al., 2009), and BAAD (Falster et al., 2015).

A European database has been developed by Zianis et al. (2005), while Forrester et al. (2017) developed one for North American tree species. Jenkins et al. (2003) provide allometric equations, and Chave et al. (2014) provide a generic model for tropical forest trees. The international platform GlobAllomeTree can be consulted for different tree allometric equations (see Henry et al., 2013 and references therein). Allometric relationships are derived from a subset of trees. AGB observation plots often relate to one tree or a smaller group of surrounding trees. Trees have to be marked or exact coordinates are needed. Guidelines on the selection of trees can be found in Smart et al. (2017) and Bertora et al. (2012).

Allometric equations are highly species specific and thus accommodate species-specific and individual responses to climate manipulation. The use of published allometric relationships for a species may be suitable if the development of AGB over time is of interest because the incremental change in AGB over a given time rather than the actual measure of AGB is the information of interest. However, if the environmental conditions between years are very different and the responses of trees to these conditions change, the desired information in AGB may be hidden in the “noise” introduced by using non-specific allometric relationships (Coomes & Allen, 2007).

If AGB of small forest stands or individual trees is of interest, we suggest building allometric relationships from nearby trees of the species of interest (see Bertora et al., 2012 for guidelines). The building of allometric relationships requires either the sampling of branches or the felling of trees

(Monserud & Marshall, 1999), and the actual measurement of weight, DBH, and wood density. If such sampling is not possible, a protocol involving tree coring and seasonal litter sampling as introduced in Smart et al. (2017, supplementary material) is preferred over the use of general allometric relationships found in the literature.

Handling and storage of AGB (Gold standard)

Plant material should be stored in a cool place if no oven is immediately available as metabolic processes can continue. All plant material is dried at a maximum of 70 °C for 72 h to constant weight. Dried plant material shall be stored in a desiccator during cooling down to prevent the dried material taking up air humidity. Dried and cooled plant material is weighed to the nearest 0.01 grams. AGB is expressed as weight of dry matter per unit area (g AGB m^{-2}).

There is no clear rule, what the optimal temperature for drying biomass is. Drying temperatures range from 50 - 80 °C (NutNet protocol; Milner & Elfyn Hughes, 1970; Pérez-Harguindeguy et al., 2013). Dependent on the plant material (fleshyness, thickness), the time plant material needs to dry to constant weight varies. Too high temperatures can change components in the plant (e.g. damage secondary compounds, carbon compounds), which will affect the biomass. Contrary, if biomass is dried at too low temperature (50 - 60 °C), metabolic processes may continue during the drying process or can trigger a stress reaction in the plant. In both cases this will affect the biomass of the plant.

Dried AGB can either be stored in paper bags, or stored frozen in plastic bags. The latter is more convenient if samples are to be sorted into different fractions. If possible, dried material is measured for carbon and nutrient content.

Where to start

Chave et al. (2014), Damgaard (2014), Fahey & Knapp (2007)

2.1.1.2 Special cases, emerging issues, and challenges

a) Special case: AGB sampling in grazed systems

To measure AGB in grazed systems, the gold standard is the “moveable exclosure” (ME) method (McNaughton et al., 1996; Knapp et al., 2012). It implies a paired plot design, where grazed study plots are combined with adjacent ME plots. The paired plots are moved one to several times per year. The movement frequency and the repeated sampling of plant biomass should reflect both the intensity of herbivory and plant re-growth rates (McNaughton et al., 1996); in semi-arid environments, monthly time intervals during the growing season have been used (Knapp et al., 2012). Prior to randomly moving the paired plots to a new location within the treatment area, AGB is harvested both on the ME plots (or from a smaller area within the ME) and on the adjacent grazed plot. In this special case, ANPP can be calculated from AGB harvested in ME plots (see below) while the AGB from grazed plots is used to estimate grazing offtake (Linstädter & Ruppert, 2015).

The bronze standard for measuring AGB under grazed conditions is the "clipping method". Grazing exclosure cages are left in the field preventing actual grazing. Vegetation in exclosure cages is simulated via clipping, with the end-of-season standing crop also being clipped (McNaughton et al., 1996). The clipping method can mimic grazing if livestock grazing is not possible, for example due to small size of treatment plots (Linstädter & Ruppert, 2015).

b) Timing of sampling

Sampling can be done at different times during the season to address different ecological questions. We advise that sampling dates are not fixed but are flexible to accommodate the phenological stage of the plants, and matched with management practice if necessary. Moreover, biomass sampling has to be attuned to vegetation characteristics (herbaceous v. non-herbaceous, patchy v. non-patchy), and to the seasonality of the ecosystem (Linstädter & Ruppert, 2015). To quantify maximum biomass, AGB is often sampled at peak growing only (NutNet protocol; Linstädter & Ruppert, 2015). To quantify sequential growth, biomass is harvested at several times during the growing season (Scurlock et al., 2002). This "repeated sampling" is the gold standard for systems with a marked seasonality, and particularly for systems with high biomass turnover rates, such as temperate and humid environments (Scurlock et al., 2002; Ruppert & Linstädter, 2014).

c) Standing and attached dead biomass

The term "biomass" may be defined to include standing dead trees and dead branches on live trees. Dead branches can be assessed using standard procedures (Monserud & Marshall, 1999). However, because they may lack small branches and foliage, standing dead trees often require different model forms and parameterisation (Powers et al., 2013). In either case, dead trees and branches must be treated carefully because they can represent a significant biomass pool.

d) AGB conversion to Aboveground Net Primary Production (ANPP, grams of biomass per m^2 per year)

The method used to convert AGB measurements to ANPP should reflect the AGB estimation method, i.e. if AGB was harvested several times during the growth period, ANPP is estimated with "incremental methods", which sum the seasonal accumulation of biomass (Ruppert & Linstädter, 2014; see Scurlock et al., 2002 for details on the protocols). We suggest the use of Smalley's method (the sum of positive increments in live and recent dead biomass; Method 5 in Scurlock et al., 2002) for ecosystems and experiments where biomass can be destructively harvested as it is a good compromise between accuracy and sampling effort.

Alternatively, AGB can be converted to ANPP using the sum of positive increments in living biomass only (Method 4 in Scurlock et al., 2002). This method is similar to AGB conversions to ANPP that are used for non-destructive estimations of green AGB (see above). As AGB from non-destructive measurements is performed at peak growing season, the conversion of AGB into ANPP can be done with a "peak method" that uses single biomass measurements at peak biomass conditions (Ruppert & Linstädter, 2014; see Scurlock et al., 2002 for details on the protocols). Among these peak methods, "peak standing crop" (live plus recent dead biomass) is the gold standard. To calculate ANPP using the peak method, AGB estimates from two consecutive years are needed to calculate ANPP as:

$$ANPP\ (g\ biomass\ m^{-2}\ yr^{-1}) = AGB_{year\ x} - AGB_{year\ x-1}$$

For annual plants, AGB equals ANPP. For perennial plants, including trees, a carbon-budget approach is necessary. Such an approach estimates ANPP from:

ANPP = Change in aboveground biomass + mortality + litterfall

An alternative is to focus on woody ANPP, which allows one to use the change in biomass estimated from allometrics (Gough et al., 2013), but it remains necessary to account for any mortality that may have occurred.

e) Light Detection And Ranging (LIDAR), can be used to derive biome specific AGB estimates (for details see protocol 2.3.3 Upscaling from the plot scale to the ecosystem and beyond).

2.1.1.3 References

Theory, significance, and large datasets

Henry et al. (2013), Kattge et al. (2011)

More on methods and existing protocols

Beierkuhnlein (2006), Bertora et al. (2012), Mueller-Dombois & Ellenberg (1974), NutNet protocol, Scurlock et al. (2002)

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2.1.2. Belowground plant biomass

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Measurement unit: g roots/soil volume or root length; Measurement scale: plot; Equipment costs: € - €€€; Running costs: €; Installation effort: medium; Maintenance effort: low to medium; Knowledge need: medium; Measurement mode: manual

A large proportion of plant biomass is located belowground, increasingly so towards the poles. For example, on a biome scale the root:shoot ratio ranges from 4.8 in tundra to 0.2 in tropical forests (Mokany et al., 2006). Besides anchoring the plants, roots also provide the plants with nutrients either through passive uptake via the mass flow of water or through active ion uptake, which requires some of the carbon allocated to roots. As much as 25–75% of the carbon assimilated by aboveground biomass (ABG) is transferred belowground and used for the production of roots, their associated rhizobiome, and respiration. About half of net assimilated C is used for respiration (Marschner, 1995; Gill & Jackson, 2000). Up to 30% may be transferred to the soil as root exudates (see protocol 2.1.5 Root exudation), and associated symbionts such as mycorrhiza also require a substantial part (see protocol 2.2.2 Root and soil colonisation by mycorrhizal fungi). Similar to the AGB, roots also have litterfall through sloughed cells, dead roots, and root exudates. Often, annual root litterfall has been equated with annual root net primary production in C budgets (Leppälammi-Kujansuu et al., 2014) but data are sparse.

The assessment of belowground biomass (BGB) provides important information on ecosystem carbon and nutrient storage and cycling but is – in contrast to AGB – difficult to assess. Accurate field-based measurements of BGB across ecosystems are needed to improve current regional-scale models for belowground carbon sequestration and carbon cycling through the turnover of fine roots. Measurements of roots are mostly very intrusive and in climate-change studies non-intrusive methods are needed to avoid destruction of the studies. Often BGB has been assessed as a fixed fraction of AGB but this relationship may change considerably along environmental gradients and in response to climate change and other global-change drivers. The measurements can be challenging as the biomass varies across the soil profile, as well as different root classes (size and orders) having different functions, plus it can be difficult to distinguish between roots of different species or if a root is dead or alive.

Although there is no consensus in the literature on how to estimate root biomass, production, and turnover most accurately (review by Addo-Danso et al., 2016), several direct and indirect methods are available to measure biomass and production of fine and coarse roots (Hendricks et al., 2006; Addo-Danso et al., 2016).

2.1.2.1 What and how to measure

a) Root biomass

Gold standard

The most common method to assess fine-root biomass is direct harvesting of roots from soil-cores (Ostonen et al., 2005). For coarse roots, direct methods include root excavation in soil-pit, wall, or trench profiles and soil-coring. Direct excavation techniques by picking only fine roots in the field tend to underestimate fine-root biomass by about 40% due to loss of fine roots during excavation (Rosenvald et al., 2014).

Root biomass and root production from soil coring

Soil coring. The most common approach determining fine-root biomass in the field is the soil coring method (see Picture 2.1.2.1) in which a soil sample is taken with a soil corer (typical diameters range from 3 to 10 cm) to the relevant depth, often the main rooting depth. It is important to measure depth and diameter of the corer for calculation of root biomass on a unit area and soil volume basis. Fine-root biomass estimates from soil cores and soil pits are closely related, and all methods should include sufficient sampling depth and sufficient replication to give robust estimates about BGB. Adequate soil sampling criteria for good estimates of BGB have been defined by Mokany et al. (2006).

Soil sampling is done with a sharp root corer (common diameter from 3 to 10 cm) from the top mineral soil down to the desired depth (e.g. 30 cm). In case there is an O-horizon, this is initially sampled on an area basis and the depth of the horizon is measured in, for example, five places.

Each soil core sample is sliced into different layers or horizons. Remember to note the depth of each layer. Soil samples for root biomass estimates can be transported in a cooler. If the roots are also used for delicate analyses as enzymatic activity or microbiome, it is recommended to use dry ice storage during field work and transportation to the laboratory. Before further processing, the adhering soil particles are washed off. Roots are separated from the soil by sieving the soil, or sorting the roots from the soil by hands or forceps, which are all laborious methods. Washing of the soil over a sieve is also possible but some of the fine roots may be lost through the sieve. Further, if the soil contains a lot of organic debris, washing or sieving is not recommended.

The root samples can be stored (at 4 °C) for shorter

Picture 2.1.2.1 Root sampling with the soil coring method in forests. The O-horizon is first sampled within the wooden frame (25 x 25 cm) down to the top of the mineral soil and the height of the O-horizon is measured at least at 5 positions along the edge of the frame. Next, soil and roots are sampled, (here from 0-30 cm with a 5 cm diameter auger. The same soil pit can be used for installation of root ingrowth cores. (Photo: Inger Kappel Schmidt)

periods before processing or frozen fresh in soil (-20 °C) for longer periods. The fresh root sample can be further divided into species or live and dead roots on the basis of colour, elasticity, and toughness (Persson, 1983). This is very laborious and it may not be possible to separate species, or live and dead roots on physical appearance and staining may be needed (Gregory, 2006). Finally, the roots are dried at a maximum of 70°C for 72h to constant weight (Pérez-Harguindeguy et al., 2013) and then weighed. The temperature and time for drying depend on the study question, how many samples are dried, the size, thickness and type of the plant material (e.g. large, fleshy or succulent leaves need more time) see protocol 2.1.1 Aboveground plant biomass for more details on the drying.

Root biomass from rhizotrons. In long-term manipulation experiments, destructive sampling of BGB is not possible as it will ruin the experiments within a few years and non-destructive or less intrusive sampling is required. Mini-rhizotrons enable continued assessment of (relative) root biomass.

Generally, there is no easy way of converting root length observations from mini-rhizotrons to standing root biomass. To convert root length from images to standing biomass, a conversion factor must be developed, as well as some assumptions regarding depth of view (of the scanner). A depth of view of 2 - 3 mm has been used although this is an arbitrary number. Furthermore, to upscale root length to root biomass requires an estimate of the specific root length (SRL, m g^{-1}). This SRL should be obtained from a destructive root sampling from the site.

Bronze standard

Indirect methods are often used for assessment of belowground biomass in larger rooting systems (e.g. trees) and include modelling, size-mass allometric equations, root:shoot ratio, or Ground-Penetrating Radar (GPR); see Addo-Danso et al. (2016) for details. In a manipulation experiment, good knowledge of treatment effects on the root:shoot ratio is required.

b) Root production

While root biomass estimates give a good indication of the belowground biomass pools in a given ecosystem, the biomass does not only include short-lived, adsorptive roots but also stable, transport roots and thus does not allow estimations on the amount of carbon that is invested into the production of new roots each year or the production of root litter. Fine-root production and turnover represent about 25% of terrestrial net primary production globally (McCormack et al., 2015). The following methods are commonly used to estimate annual production of fine roots, and each of them has their own set of advantages, disadvantages, and biases (see Table 2.1.2.1).

Table 2.1.2.1 Overview of methods to measure and estimate root biomass, production, and turnover. The table indicates destructive or non-destructive sampling approaches, sample size, and a general assessment.

	Destructive	Non-destructive	Sample size	Comments
Root biomass	Soil coring		5–10 per treatment	Soil can be used for other purposes to minimise disturbance
		Allometric models		Forests
				Can be calibrated outside the plots
		Mini-rhizotrons		Only relative root biomass
				Destructive under installation
Root production	Sequential soil coring		5–10 soil cores are sampled throughout the year	Simple but very destructive
	In-growth cores		Number depends on spatial and temporal resolution	Hole can be re-used
			e.g. 10 per treatment	
	Mesh		As for in-growth cores	Very destructive as the mesh is sampled after incubation by cutting 5–10 cm on each side of the mesh
		Mini-rhizotrons	5–10 mini-rhizotrons per treatment	Laborious but data on root production, phenology. Long-term studies
Root turn-over	Root biomass/root production		5–10 soil cores	If both root biomass and production are available, the root turnover can be calculated
		Mini-rhizotrons	5–10 mini-rhizotrons per treatment	Long-term studies possible
		^{14}C radiocarbon		Expert knowledge to sample and interpret data

Destructive methods

Sequential soil coring. A common approach of determining fine root production in the field is the sequential soil coring method (Persson, 1978; Vogt et al., 1998), where soil cores are taken repeatedly over a year. As rooting patterns are spatially variable, many replicates are needed to get good estimates on root production over time. Most commonly 5–20 samples are collected randomly or along

environmental transects in a study site at intervals of about 2–6 weeks throughout a year (more frequently during the growing season). Sequential soil coring can be used for studying biomass and how roots are distributed with depth and changes over time, but as this requires many destructive samplings, the method is laborious. For **Sample processing** see *Soil coring* above.

In-growth cores. Net root productivity can be estimated by in-growth cores (Ostonen et al., 2005; Brunner et al., 2013; Arndal et al., 2018). In-growth cores are soil pits with a mesh bag or net filled with root free soil, ideally native to the site. Thus, prior to installation, a soil core is taken and roots are removed from the soil. The mesh bag with a diameter slightly larger than the soil corer is placed in the soil pit and the root free soil is filled back into the pit. The soil can be divided into depths and filled back in the same order separated with small pieces of mesh bag material. Alternatively, the soil pit can be lined with a stiff mesh material "garden mesh/netting". A commonly used mesh size is 2 mm. The in-growth core is incubated in the soil at the same depths as the soil sampled (e.g. 0–30 cm) depending on the main rooting depth. Commonly, in-growth cores are installed vertically for practical reasons, but an angled installation may be more suitable depending on root architecture (Milchunas, 2009).

During harvesting, roots outside the bag are removed with scissors after which roots from the bag can be picked with forceps and washed. The harvesting frequency is biome and plant species or community specific. In boreal forests and sub-arctic regions, where annual root production is low, the in-growth cores should be harvested for at least two or three consecutive years and 2–3 times a year for annual production (Makkonen & Helmisaari, 1999; Ostonen et al., 2005). However, in the tropics and temperate ecosystems, and for pioneer plant (tree) species, shorter time periods are appropriate. After the first harvest, the root-free soil may be replaced in the pit and a subsequent harvest can take place. Installation in early spring and late autumn will enable assessment of root production in winter and in the growing season, but species- and biome-specific differences have to be taken into account to avoid underestimation. In-growth cores require more preparation time than sequential soil coring, but give more accurate estimates of root production over time. As the root-free core needs to be re-colonised, there is a risk of under- or over-estimating root production depending on root competition, and in-growth cores generally give a lower estimate of root production than mini-rhizotrons and isotope turnover methods (Milchunas, 2009). This risk can be reduced by using smaller diameter cores and more replicates. For example, Laiho et al. (2014) showed that 10–30 cores (with a diameter of 3.18 cm) were needed to get robust estimations of root production in peatland forests and a fen. In manipulation experiments, the relative root production rate may be sufficient. For **Sample processing** see *Soil coring* above.

Root in-growth mesh (called also nets, sheets, screens)

Measurements of root production by sequential soil coring, root imaging (see below), or by in-growth cores are time and labour intensive, have a high degree of uncertainty, and can induce changes in the chemical and physical properties of the soil. The root in-growth mesh method has been proposed as an alternative technique to overcome these problems (Fahey & Hughes, 1994; Jentschke et al., 2001; Godbold et al., 2003). Using this technique, root production is estimated by placing a mesh (for example 7–10 cm x 20–40 cm, 1–2 mm mesh size) vertically into the soil for a specific period of time and then

measuring the number and weight of roots that grow through the mesh (Hirano et al., 2009). The procedure is much easier than other methods, requires only simple equipment, and causes minimal soil disturbance (Godbold et al., 2003). The method has been discussed by Ohashi et al. (2016) and Montagnoli et al. (2014).

The root nets (preferably 15–30 per sampling time) are extracted from soil as 10 x 10 cm soil blocks (Lukac & Godbold, 2010) annually at the end of every vegetation period. Extracted nets are washed free of soil in the lab, and fine roots grown through the mesh are counted and cut to 1 cm on both sides of the net to create a 'virtual' 2 cm thick core. Roots are removed from the net and different plant species can be separated if visually distinguishable, although only living roots can be estimated by this method.

Several methods exist to calculate fine-root production from the change in the fine-root biomass and necromass data. The "Maximum-Minimum" method calculates the annual fine-root production by subtracting the lowest biomass from the highest biomass value irrespective of other biomass values recorded during a full year (McClaugherty et al., 1982). The "Decision Matrix" method calculates the annual fine-root production by balancing the living and dead root biomass compartments and summing all calculated productions between each pair of consecutive sampling dates throughout a full year (Fairley & Alexander, 1985).

Non-destructive methods

Root imaging: Mini-rhizotrons, flatbed scanners, smartphone solutions. The most common technique based on direct observations of root growth dynamics *in situ* is the mini-rhizotron method (see Picture 2.1.2.2), although there are also root windows, rhizotrons, and flatbed scanner or smartphone imaging methods in use. All methods enable following root growth dynamics and phenology from birth to death of the finest, ephemeral, and distal roots (as reviewed in Hendrick & Pregitzer, 1996; Johnson et al., 2001; Rewald & Ephrath, 2013; Mohamed et al., 2017).

Photo 2.1.2.2 Installation of a mini-rhizotron. (Photo: Inger Kappel Schmidt)

First stage is the installation of permanent transparent tubes of c. 5–7 cm in diameter (mini-rhizotron) or boxes for flatbed scanners or smartphone cameras into the soil (Mohamed et al., 2017), or root windows that are soil profiles covered by transparent plexiglass plates (Stober et al., 2000).

Mini-rhizotron installation is usually done using an angled guide and soil corer (Johnson et al., 2001, Rewald & Ephrath, 2013). This is typically at a 45 ° angle (for observations at different depths while ensuring good capture of vertical and horizontal rooting systems) or horizontally (for more detailed observations at a

certain depth of interest; Smucker, 1993). After installation, some time has to pass before the first measurements to allow the vegetation to recover from the disturbance. The time needed depends on the natural stability of the system and species present, but an inactivity phase of 6–12 months is common (Rewald & Ephrath, 2013).

For root imaging, a tube-fitting camera or scanner is inserted into the tube to take pictures of the tube-soil interface (about 3 mm space behind the tube class), where the roots grow. By repeatedly taking pictures of the same spot (ensured by an indexing handle on the camera system), root production and mortality over time can be measured. A crucial aspect is to choose the time interval between these measurements, as there is a trade-off between availability of time and resources and measurement accuracy (Johnson et al., 2001). Long sampling intervals can underestimate root production, as roots can grow and die off between sampling events, and, in general, relatively shorter intervals should be used for more productive systems. Most studies use intervals of 2–4 weeks: using intervals of < 3 weeks did not underestimate root production in five different ecosystems (Balogianni et al., 2016).

Sample processing: The method is very labour intensive. Currently, the images of the roots have to be analysed manually by mapping all roots, usually using specific software (WinRhizotron (Regent Inc), Rootfly (Clemson University, free software), RootSnap (CID BioScience, free software), etc).

Mini-rhizotrons are an excellent tool to measure timing of root production and mortality (root phenology), and are often used for estimating root longevity and turnover. However, they are not the best method to estimate root biomass, as they only offer a two-dimensional view into the soil. Several conversion methods exist that use mini-rhizotron data to infer root biomass: see Metcalfe et al. (2007) for a comparison of different methods.

Turnover rate

The term turnover rate of a certain root category means the rate that root biomass is replaced during a year. Fine-root turnover rate is dependent on the fine-root biomass and the annual production of fine roots, but also on the various methods and calculations (Brunner et al., 2013). The mean lifespan can be calculated by dividing the “pool” (biomass) by its “input” (annual production). Because the turnover rate is the inverse of lifespan, it can be calculated by dividing the “annual production” by the “root standing biomass” (Gill & Jackson, 2000), which most commonly means an average root biomass, but there are several studies using maximum root biomass instead. According to Brunner et al. (2013), however, using maximum fine-root biomass to calculate the fine turnover rate, results in about 30% higher values of root turnover. To decrease the lab intensity, a combined method has been proposed – fine-root turnover rate is calculated from in-growth cores/sheets and multiplied by fine-root biomass estimated from soil cores (Ostonen et al., 2005; Löhmus & Ostonen, 2006).

With mini-rhizotrons, individual roots can be observed from birth to death for estimations of root longevity. However, this is difficult for ecosystems with long-lived roots or systems with a lot of soil movement (e.g. freeze-thaw or drying and re-wetting of soils). Similarly to destructive methods, turnover rate can also be calculated with mini-rhizotrons by using the observed root growth and total root length density.

Radiocarbon (^{14}C) content has been used to estimate fine-root productivity and fine-root turnover (Gaudinski et al., 2001) using the fallout ^{14}C signal from nuclear weapons testing in the 1950s and 1960s. The method requires good knowledge and sampling of roots of known age.

Where to start

Addo-Danso et al. (2016), Mancuso (2012), Rewald & Ephrath (2013), Smit et al. (2014)

2.1.2.2 Special cases, emerging issues, and challenges

^{15}N assay. Nitrogen deficiency can be assessed in fresh roots using a ^{15}N labelling assay (Jones et al., 1991). The roots are placed in a ^{15}N solution on the same day as harvesting and ^{15}N uptake in the roots can be measured with mass spectrometry. The assay with excised roots represents a relative measurement of the root net uptake capacity and not the actual uptake rate, as would have been found with the roots still attached to the plant (Göransson et al., 2007). High ^{15}N uptake demonstrates nitrogen limitation (Jones et al., 1991; Michelsen et al., 1995; Arndal et al., 2014).

The N-deficiency bioassay follows the procedure of Jones et al. (1991). Using roots from in-growth cores, the root material will be relatively homogeneous and with a maximum age similar to the time the in-growth cores have been in the soil.

Roots are sorted by hand and fine roots washed in demineralised water. The roots are stored in a plastic bag and kept in the dark at 5 °C until the assay is conducted (best the same day as sorting).

The fresh root bundles are marked with a name tag and pre-soaked for 30 min in 5 x 10^{-4} M $CaCl_2$ solution) to maintain root cell-membrane integrity and to remove ammonium and nitrate from the cell-free space. After the pre-treatment, the roots are transferred to 5 x 10^{-4} M $CaCl_2$ containing $^{15}NH_4Cl$ (20% enrichment; 2 ml $^{15}NH_4Cl$ and 8 ml $^{14}NH_4Cl$). They are left in the solution for 2 h, washed for 15 min in running demineralised water, dried in a paper bag, weighed, and finely cut.

Analysis of ^{15}N excess

Samples of about 12 mg root are analysed on a mass spectrometer. Excess ^{15}N (atom %) can be converted to absorption rate of N (mgN g^{-1} rootDW 2 h^{-1}). The total N in the roots can be calculated, after subtraction of N which had been taken up during the bioassay (Michelsen et al., 1995).

Root:shoot ratio

Due to the destructive nature of root sampling, AGB is often used as a rough estimate of BGB. A fixed belowground:aboveground ratio is typically applied to estimate BGB from AGB (see review in Addo-Danso et al., 2016), although a meta-analysis of global data shows this to be a questionable approach (see Mokany et al., 2006) as the functional relationship between the aboveground and belowground

parts of the vegetation may change due to climatic factors, for example drought or elevation, and CO_2 may increase the R:S ratio due to water or nutrient limitation, respectively.

A number of studies report on the functional relationship between AGB and BGB, for example the needles:fine-root ratio (Helmisaari et al., 2009). Absorptive fine-root biomass (aFRB) per stand basal area (BA) (aFRB/BA, kg m^{-2}) has been used as a proxy to describe the functional relationship between the aboveground and belowground parts of a forest stand (Ostonen et al., 2011, 2017) and the use of vegetation-specific root:shoot ratios were found to be a more accurate method for predicting root biomass (Mokany et al., 2006).

Sample size and sample depth

The issue of sample volume is complicated and the same method may not be suitable for both fine and coarse roots. Different methods vary by more than an order of magnitude with respect to soil volume sampled (Taylor et al., 2013). Adequate soil sampling criteria for good estimates of BGB in a certain area have been defined by Mokany et al. (2006) as follows: the depth of root sampling has to be > 75 cm for deserts and very arid ecosystems, > 50 cm for woodlands, savannahs, shrublands, temperate and tropical forests, > 30 cm for grasslands and boreal forests, and > 20 cm for tundra. As for replication, they recommend > 10 soil cores of 5 cm diameter; > 3 soil pits 50 cm long by 50 cm wide; and entire root systems excavated for > 3 woody plants. However, the amount of replicates should also take into account how spatially variable the vegetation is, i.e. tussock tundra or shrublands may need more replicates than homogenous grasslands. In reality, few studies meet these criteria.

Upscaling

It is important to measure depth and diameter of the corer for calculation of root biomass on a unit area and soil volume basis.

2.1.2.3 References

Theory, significance, and large datasets

Freschet & Roumet (2017), Iversen et al. (2017): FRED (Fine Root Ecology Database), McCormack et al. (2015)

More on methods and existing protocols

Smit et al. (2014)

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2.1.3. Leaf-scale photosynthesis

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Reviewers: Gough C[2], Zinnert J C[2]

Measurement unit: µmol CO_2 m^{-2} s^{-1}; Measurement scale: leaf; Equipment costs: €€€; Running costs: €€; Installation effort: medium; Maintenance effort: medium; Knowledge need: high; Measurement mode: manual or automated

Through the process of photosynthesis (carbon dioxide assimilation, A), plants fix carbon dioxide (CO_2) from the atmosphere and release oxygen (O_2), harnessing light energy to produce stored chemical energy. This chemical energy is stored in carbohydrate molecules, such as sugars and starch, which are synthesised from CO_2 and water (H_2O). Measurements of photosynthesis are necessary to understand the mechanisms, magnitude, and dynamics of leaf-level carbon fixation and its relationship to plant biomass accumulation (i.e., net primary production) (Geider et al., 2001). Quantitative understanding of species- and functional-group specific relationships between the dynamic environmental drivers of photosynthetic capacity informs predictions – especially via simulation modelling – of leaf- to ecosystem-scale carbon sequestration as climate changes (Taiz & Zeiger, 2010; Kodama et al., 2011; Ashraf & Harris, 2013; Atkin, 2015). Rates of photosynthesis are sensitive to the quantity of light absorbed by chlorophyll, air temperature, soil water availability, humidity, elevated atmospheric CO_2, and soil conditions including nutrient availability (Kirschbaum, 2004). In addition to climatic drivers, other global-change drivers such as pollution in the form of ozone and SO_2 (Heber et al., 1995), fertilisation and nitrogen deposition (Evans & Clarke, 2018), and fire (Yue & Unger, 2018) influence leaf-scale photosynthesis. The methods described here can be applied to study the responses of leaf photosynthetic performance to all these different drivers. There are different approaches available for estimating photosynthesis, depending on the temporal and spatial scale of interest (Hunt, 2003; Barbour et al., 2007; Millan-Almaraz et al., 2009). The upscaling of leaf-level photosynthesis to ecosystem level is dealt with in protocol 2.3.3 Upscaling from the plot scale to the ecosystem and beyond. Most commonly, leaf-level photosynthetic parameters are measured using non-destructive, commercially available gas-exchange systems, many of which allow the operator to manipulate the environmental conditions of the leaf cuvette.

2.1.3.1 What and how to measure?

Gold standard

Carbon dioxide assimilation (A) is most commonly measured on intact leaves using a portable gas-exchange system with a climate-controlled cuvette in which light intensity, CO_2 concentration, temperature, and humidity are precisely regulated (Farquhar et al., 1980; von Caemmerer & Farquhar, 1981; Long et al., 1996; Long & Bernacchi, 2003). Leaf-level light-response curves – constructed through measurements of A at a series of light levels – provide information on several components of gas exchange, such as dark respiration rate (R_d), light compensation point, quantum efficiency (φ), and light

saturated photosynthetic rate (A_{sat}; Figure 2.1.3.1A; Ögren & Evans, 1993; Lobo et al., 2013). Photosynthetically active radiation (PAR) is the main limiting factor for A at low light intensities, while at higher light intensities other factors, such as CO_2 uptake are more limiting to A (Ögren & Evans, 1993). However, full light-response curves are time consuming. It requires up to 15–20 min measuring time at each light intensity (or 5 min per curve for rapid light response curves, although this leaves no time for stomatal adjustment; but see *Special cases, emerging issues and challenges*). The photosynthetic rate under light-saturating conditions (A_{sat}) is commonly used to rapidly evaluate photosynthetic capacity. The methodological aspects of measuring light-saturated photosynthetic rate are summarised in Pérez-Harguindeguy et al. (2013), where the term A_{max} is used instead. We note that A_{max}, A_{sat}, and light saturated A_{net} are often used synonymously to indicate light-saturated photosynthetic rate. However, Jarvis & Davies (1998) defined the maximum rate of photosynthesis, A_{max}, as A_{sat} when CO_2 is not limiting, a condition rarely met under ambient atmospheric CO_2 concentrations. In most models, the A_{net} term of light saturated is commonly used (Damour et al., 2010).

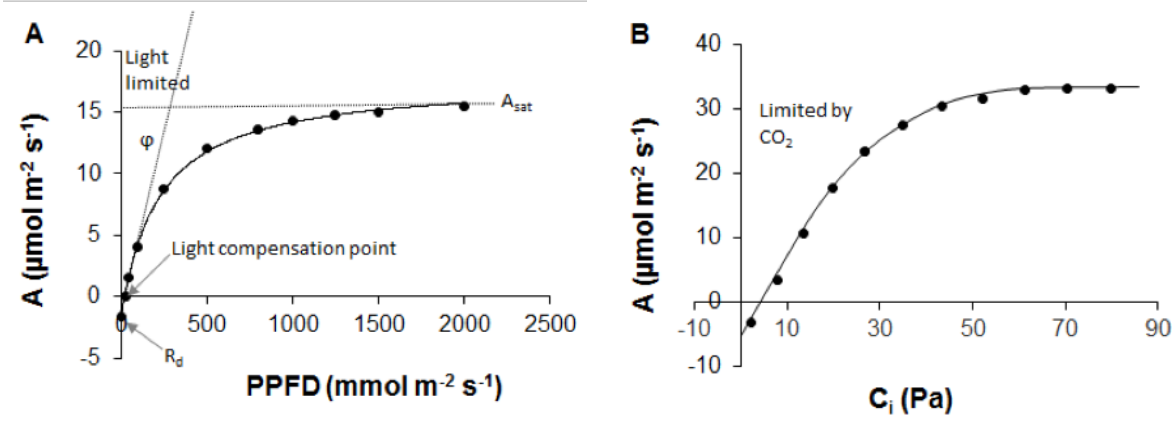

Figure 2.1.3.1 **A** Leaf-level light-response curve (solid line) showing the response of the net photosynthetic rate (A) to photosynthetic photon flux density (adapted from Lobo et al., 2013). Dark dots are for observed A. Slope (φ) of the curve shows maximum apparent quantum efficiency of the leaf (how efficiently solar energy is converted into chemical energy), R_d is the dark respiration rate, light compensation point is the light intensity at which respiration equals photosynthetic rate, A_{sat} is the light-saturated photosynthetic rate. **B** A/C_i curve showing the response of net photosynthetic rate (A) to intercellular CO_2 partial pressure (C_i). Dark dots are for observed A (data derived from Sharkey et al., 2007).

One might also measure the response of CO_2 uptake to intercellular mole fraction of CO_2 (termed A/C_i) (Figure 2.1.3.1B) to separate the biochemical and stomatal limitations on photosynthesis (Long & Bernacchi, 2003). C_i refers to intercellular CO_2 concentration. Intercellular CO_2 is the main limiting factor for A only at low CO_2 concentrations, while at higher CO_2 concentrations other factors set limits to A as well (Long & Bernacchi, 2003). The A/C_i response is determined by measuring A and transpiration at a series of ambient CO_2 concentrations (C_a) and a defined leaf-air vapour pressure deficit (Long & Bernacchi, 2003).

Bronze standard

When a climate-controlled chamber is not available, one can make measurements of A under ambient conditions. Or, if an actinic light source is available, light saturation may be achieved without controlling for additional environmental factors known to influence A. These do not allow for analysis of metabolic controls of photosynthesis, but they do provide a survey of the breadth of A in response to variable conditions and may be well-suited to studies focused on the degree of inherent spatial and temporal variation in A.

Timing

The timing of a measurement is an important factor to consider. Measurements intended to derive maximum photosynthetic capacity should, ideally, be taken during the period of maximal photosynthetic activity of leaves, usually mid to late morning (Pérez-Harguindeguy et al., 2013), avoiding the period of afternoon depression when water status causes stomatal closure and thus limits CO_2 supply. However, depending on location and species, the timing of maximal photosynthetic activity might differ. A_{sat} should be measured during peak-growing season as timing of leaf-out and senesce can vary by species and if not considered could skew the results (Lim et al., 2007). For the calculation of whole day carbon assimilation of a plant, assimilation (A) at ambient light can be monitored from sunrise until sunset to calculate the daily sum of assimilated carbon, but it is very time-consuming and, for extrapolation over time, needs to be calibrated over different seasons and temperature conditions. Models can be applied to integrating over time and scaling across space leaf level A (see e.g. Patrick et al., 2009).

Selection of leaves

Commonly, light-saturated and fully developed canopy leaves are selected to allow for inter-specific species comparison of maximum photosynthesis rate, unless the aim is to compare the photosynthetic properties of leaves from different light conditions. This is relevant also in cases when you use an internal light source and adapt the leaf to saturating light as not all parameters (such as leaf structure, chlorophyll content etc.) which change under different light conditions and determine leaf photosynthetic rate, change during the minutes of acclimation. This is especially important for plants with thick leaves (Niinemets, 1999). With higher canopies (e.g. trees), it is important to consider the steep gradient in light conditions along the canopy when extrapolating leaf-level photosynthesis to whole-canopy photosynthesis (Niinemets et al., 2004). For narrow (e.g., needleleaf) leaves, several thin leaves can be placed side by side in the leaf chamber. If the measuring area is not fully covered with a leaf or leaves, considerable bias in the results may occur unless the surface area measured is adjusted. Commercial gas-exchange systems often provide special cuvettes or allow the adjustment of the sampling area.

Measurement conditions

Depending on the research question, one may use ambient environmental conditions v. more tightly controlled conditions. However, gas exchange measurements should not be done during windy weather or with wet leaves. For the measurements of A_{sat} the light conditions must be saturating for at least 5–10 min prior to measurement (Pérez-Harguindeguy et al., 2013). In some cases, species with high sensitivity to water status will decrease photosynthetic activity following even a minor drought and thus, time since last rainfall should be considered where necessary (Medrano et al., 2002). Ambient CO_2 concentrations are preferred, but may be manipulated when the effects of CO_2 enrichment are of interest or for A/C_i curves. In manipulation experiments, as a gold standard, one should measure photosynthesis at both control and manipulated conditions. If time is limited, then as a bronze standard, photosynthesis in manipulated conditions should be measured as a priority.

Where to start

Boardman (1977), Farquhar et al. (1980), Long & Bernacchi (2003), Ögren & Evans (1993), Sharkey et al. (2007)

2.1.3.2 Special cases, emerging issues, and challenges

When measuring needleleaf photosynthesis (such as conifers), special chambers are preferred. To calculate the photosynthetic capacity on a leaf area basis, leaf area should be presented as the average projected total needle area (Grace, 1987). Leaf mass or leaf mass per area is sometimes used as the basis of comparison for complex leaf shapes. Ecosystem-scale estimates of photosynthesis (e.g., gross primary production) have been dealt with in protocol 2.3.3 Upscaling from the plot scale to the ecosystem and beyond.

Recently, a new technique was developed that captures full A/C_i curves within 5 min (Stinziano et al., 2017), which can potentially circumvent complications involving stomatal closure, changing water potential, enzyme activation, and chloroplast movement. It should be noted that such rapid measurements do not allow time for stomata to adjust to changes in CO_2. The resulting A/C_i response is correct, but the stomatal adjustment to varied CO_2 is not captured.

Historically A/C_i curves have dominated the literature, but the maximum carboxylation rate of Rubisco (V_{cmax}) is actually controlled by C_c, the CO_2 concentration in the chloroplasts. Therefore, A/C_c curves should be preferred where possible (Sharkey, 2016). The difference between C_i and C_c is determined by the mesophyll conductance. Measurement of mesophyll conductance remains somewhat problematic (Pons et al., 2009), but intensive work is underway.

A relatively new and fast developing method used in photosynthesis research is the stable carbon isotope analysis of CO_2 (Barbour et al., 2007). The ratio of ^{13}C to ^{12}C of CO_2 entering and leaving the leaf chamber can be determined by a tunable diode laser (TDL) spectroscopy system, cavity ring-down absorption spectrometry, or quantum cascade laser. This enables the measurement of the leaf respiratory or photosynthetic CO_2 flux and its associated carbon isotope composition ($\delta^{13}CRl$). The latter

has the potential to allow partitioning of ecosystem respiration into various components (Tu & Dawson, 2005) enabling the tracing of carbon fluxes through plants and ecosystems at high temporal resolution and allowing very complex interpretations of ecosystem carbon losses (Kodama et al., 2011; Wehr et al., 2016). The stable isotope composition can also be used to infer intrinsic water-use efficiency, a measure of the trade-off between photosynthetic gains and transpiration losses (Marshall et al., 2007). Because the stable isotope ratio is determined at the chloroplast, estimates of mesophyll conductance can also increase the utility of this measurement (Wehr et al., 2016).

Chlorophyll fluorescence (see protocol 5.1 Chlorophyll fluorescence) measurements can be combined with gas-exchange measurements to study the functioning of the photosynthetic machinery in more detail and to infer the partitioning of energy between photosynthesis and respiration (Laisk & Loreto, 1996). However, the different parts of the leaf from where the CO_2 signal and fluorescence signal comes, the alternative electron sinks, and other critical aspects need to be considered for interpretation (Yin et al., 2009).

2.1.3.3 References

Theory, significance, and large datasets

Ainsworth & Long (2005), Geider et al. (2001), Reich et al. (1998), Taiz & Zeiger (2010), Zhu et al. (2010), https://daac.ornl.gov/VEGETATION/guides/Leaf_Photosynthesis_Traits.html

More on methods and existing protocols

Hunt (2003), Lobo et al. (2013), Millan-Almaraz et al. (2009), Pérez-Harguindeguy et al. (2013), Sharkey (2016)

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2.1.4. Plant respiration

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Measurement unit: µmol m^{-2} s^{-1} (or g^{-1} or m^{-3}), µmol g^{-1} s^{-1}; Measurement scale: tissue, plant, or plot; Equipment costs: €€€; Running costs: €; Installation effort: medium to high; Maintenance effort: -; Knowledge need: high; Measurement mode: manual measurement or data logger

Plant respiration is the carbon dioxide (CO_2) efflux from vegetative tissues to the atmosphere. With plants globally respiring upwards of 60 gigatons of carbon per year (Schimel 1995), this flux is one of the largest contributors to atmospheric CO_2 (Raich & Potter, 1995). Plant respiration converts roughly half of fixed photosynthate back into CO_2 (Lambers et al., 2008; Amthor, 2010). The difference between photosynthesis and plant respiration represents net primary production.

Respiration occurs in all living plant tissues whether in darkness or in sunlight, but rates are especially sensitive to kinetic changes owing to temperature and tissue growth rates, increasing exponentially with temperature and linearly with growth rates. Climate and other global-change drivers such as elevated CO_2 and nitrogen depostion can influence temperature and growth rates and thus respiration rates (Ryan, 1991; Hyvönen et al., 2007).

2.1.4.1 What and how to measure?

Gold standard

The sensitivity of the respiration rate to dynamic environmental conditions and its coupling with plant growth necessitates high frequency, continuous measurements if the goal is to disentangle the underlying drivers regulating plant respiration rates. For this reason, the gold standard is continuous measurements of CO_2 efflux (Tarvainen et al., 2014). This requires either closed or open chambers mounted on or around the plant organ of interest (Field et al., 1989; Tamayo et al., 2001, *see Special cases below*). Closed chambers must be opened between measurements using some automated mechanism. Open chambers can remain closed, but they require a cooling mechanism if they are exposed to daytime sun. In theory, O_2 influx could also be measured (Gonzalez et al., 2007), but in fact this is seldom done in terrestrial ecosystems because the background concentration in the atmosphere is so high that a change would be difficult to detect.

Bronze standard

The bronze standard is to use a portable gas-exchange system to take periodic point measurements manually at intervals. At its most basic, such a measurement can be treated as an index to be used as a basis for comparison within a study. However, due to the less frequent nature of manual measurements relative to those that are automated, and the potential for changing environmental conditions to bias

measurements across plots and sites, manual measurements are generally less suitable for integrative estimates of whole-ecosystem plant respiration. Some standardisation of manual data collection (e.g., by time of day and season) should be considered in order to reduce environmental bias and the effects of co-occuring photosynthesis on plant respiration.

For climate-change experiments, it may be more useful to use manual spot measurements to calibrate, or to compare to general models that were parameterised elsewhere (e.g. Heskel et al., 2016). If annual scale summations are required, they can be derived from response functions scaled up using continuous abiotic data (e.g. Niinistö et al., 2011).

Also see protocol 2.2.3 on Soil CO_2 and trace gas fluxes for more details on measuring gas fluxed from the soil.

Where to start

Start with Ryan's description (Ryan, 1991), which is clear and interesting. Then read Heskel et al. (2016) or Reich et al. (2016) for a look at global patterns. If you want more of the physiology, Amthor (2010) is a good read.

2.1.4.2 Special cases, emerging issues, and challenges

Continuous measurements can be made with either open or closed chambers (Field et al., 1989; Tamayo et al., 2001). Open chambers rely on a continuous flow of gas and the measurement of the CO_2 concentration difference between the inflow and the outflow. Open chambers have low sensitivity to leaks, but they require careful measurement of flow rates. Closed chambers rely on a timed mechanism to seal the chamber, followed by measurement of the rise in concentration over time, and then opening of the chamber after the measurement. Such chambers are sensitive to leaks and temperature increases during the measurement. In either case, chamber temperatures should be measured with some precision, especially if the chamber is in bright sunlight. Still better is some form of chamber cooling system, which prevents the temperature rise that would otherwise occur.

Continuous respiration measurements often rely on a commercial photosynthesis system that has been modified for continuous operation. Spot measurements can be made with most commercial gas-exchange systems provided that enough living tissue can be fitted into the chamber to yield a reliable measurement. Because respiration rates are often 10% or so of net photosynthetic rates, the measurement can challenge traditional gas-exchange systems, especially at low temperatures.

Respiration rates can be expressed relative to tissue mass, surface area, or volume. The best basis depends on the study objectives, but mass is often easiest to measure and is well correlated with respiring tissue quantity. However, there is a strong tradition of expressing leaf respiration per square metre (Heskel et al., 2016). In the interest of generating comparable data across studies, we suggest using mass as the default basis for all rates except for leaves, which should be expressed per m^2.

When leaves or green stems are sunlit, respiration may be partly or completely offset by simultaneous photosynthesis. Photosynthetic stems may either refix respired CO_2 internally, under the bark, or they may take CO_2 from the surrounding atmosphere. For the former, it seems reasonable to measure with transparent chambers, which allow refixation to proceed as normal (Cernusak & Hutley, 2011). The latter should be treated as an alternative site for net photosynthesis and measured alongside the leaves (see protocol 2.1.3 Leaf-scale photosynthesis).

Excised tissues are sometimes used for respiration measurements. This greatly simplifies chamber insertion, but the cutting may induce artefacts. In particular, "wound" respiration may be induced by the cell damage and diffusion paths are disrupted. These effects can be minimised by waiting until the pulse of wound respiration recedes or by coating cut surfaces in liquid paraffin (Cernusak et al., 2001), respectively.

It should be recognised that plants acclimatise to changes in temperature, which tends to reduce respiration responses relative to measurements in a short-term temperature experiment (Reich et al., 2016). What this means is that short-term responses should not be scaled up into seasonal or annual responses. Leaves that develop under a new temperature regime are more able to acclimatise to that regime, so the best policy would be to focus on leaves produced since the temperature treatment began (Slot & Kitajima, 2015).

2.1.4.3 References

Theory, significance, and large datasets

Theory in Amthor (2010). Significance in Amthor (2010), Heskel et al. (2016), Hyvönen et al. (2007), and Slot & Kitajima (2015). Large datasets in Heskel et al. (2016)

More on methods and existing protocols

Cernusak et al. (2001), Field et al. (1989), Heskel et al. (2016), Tarvainen et al. (2014)

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2.1.5. Root exudation (*in situ*)

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Reviewer: Preece, C[4]

Measurement unit: minutes, hours, days; Measurement scale: length, mass or surface unit of root; ground unit of a plot or site; Equipment costs: €-€€€; Running costs: €-€€€; Installation effort: high; Maintenance effort: low to high (frequent recording); Knowledge need: medium to high (species knowledge at the root level); Measurement mode: manual

Root exudation is part of rhizodeposition and a source of soluble organic carbon (C) released by plant roots to the soil. Root exudates are easily-degradable, low molecular weight organic (LMWO) substances that influence the size and sink strength of soil organic matter (SOM), since (i) they are preferentially used by soil microbes as substrate, which stimulates these microbes to also decompose less bioavailable soil organic C via a priming effect (Kuzyakov et al., 2000; Phillips et al., 2011, 2012; Meier et al., 2017), (ii) they are a chemical signal for symbiosis with mycorrhizal fungi which increase the accessibility of nutrients, (iii) they induce enzymatic degradation of SOM, and (iv) they increase soil aggregation. Exudates can be released passively due to the concentration gradient between root cells and soil solution, but they can also be secreted actively (e.g. via anion channels and vesicle transport) in response to metal toxicity, nutrient stress, the occurrence of plant and microbial taxa, and other environmental factors (Marschner, 1995; Jones et al., 2004; Bais et al., 2006; Canarini et al., 2019). The major components of root exudates are organic acids, carbohydrates, amino acids, and phenolics (Neumann & Römheld, 2007). The C release via root exudation is supposed to represent between 1 and 10% of the net assimilated C (Grayston et al., 1997; Jones et al., 2004), but may increase to up to 30% with drought (Liese et al., 2018). Under climate change, enhanced significance is attributed to root exudation, as the inducement of microbial priming effects by increased root C exudation is assumed to delay progressive N limitation (*sensu* Progressive N Limitation hypothesis; Luo et al., 2004) in forests exposed to elevated CO_2.

Methods for the collection of root exudates have developed along two main trajectories: a (semi-)artificial experimental trajectory that attempts to have sterile collection conditions for the understanding of mechanisms, and an *in situ* ecological trajectory that attempts to cover the natural complexity of root-rhizosphere interactions, for example in mature forests, and obtains the most representative exudation pattern. In the context of climate-change ecology and other global-change drivers, the *in situ* methods are of greater importance and, thus, we focus our review on these methods only. The described methods are applicable for site-based studies in the field, along environmental gradients in natural landscapes or with modified land-use or nutritional status, as well as in climate-change manipulation studies including pot experiments in a greenhouse and/or chambers. For an overview of laboratory methods the reader is referred to the literature (e.g. Luster & Finlay, 2006; Neumann & Römheld, 2007; Vranova et al., 2013; Oburger & Schmidt, 2016).

2.1.5.1 What and how to measure?

a)* In situ *root exudate collection

Culture-based cuvette method. The collection of total root exudation can be conducted with the culture-based cuvette method, which is a modification of hydroponic sampling and was adapted by Phillips et al. (2008) for field use. However, the method can be used in non-sterile greenhouse and climate chamber experiments (Gargallo-Garriga et al., 2018). In contrast to hydroponic sampling, the sampled fine root systems (terminal fine root systems < 2 mm in diameter with laterals) grow in soil and remain attached to the plant during the whole sampling process. Extraction of the fine root systems from soil has to be extremely carefully conducted to maintain the integrity of the (mycorrhizal) root system as much as possible. For this purpose, the root systems are excavated with soft brushes and fine forceps from the wall of a small soil pit and the soil particles adhering to the root system are carefully removed using deionised water, 0.5 mM $CaCl_2$, or autoclaved dilute nutrient solution. At this point of the sampling process, the species identity of the root system has to be confirmed with a morphological key (or by tracking the root system back to the mother tree). The root systems are placed overnight in moist, sandy soil to allow recovery from the excavation stress. On the next day, the soil-free and cleaned intact root systems are placed into a sterile cuvette (preferentially a glass syringe or alternatively a plastic syringe from which the plunger is removed), back-filled with sterile glass beads (≤ 2 mm in diameter) that provide the mechanical impedance and porosity of soils, and moistened with sterile, dilute nutrient solution (see 2.1.5.2 Special cases, emerging issues, and challenges below for a discussion of the culture medium). The cuvette is closed with a rubber septum and covered by plastic paraffin film, while ensuring the integrity of the emerging root. Sterile cuvettes with glass beads and culture medium (i.e. without roots) are treated similarly and included as controls. The experimental set-up is covered, for example by leaves from the litter layer to dampen temperature differences to the surrounding soil, and roots are allowed to equilibrate in the cuvette environment for 48 h. Subsequently, cuvettes are cleaned by being flushed 3 to 5 times with the culture medium using a low-pressure vacuum. New culture medium is added and the root systems are equilibrated for a sufficient time period (see 2.1.5.2 Special cases, emerging issues, and challenges below for a discussion of the incubation time). After the incubation period, the trap solutions containing exudates are collected from each cuvette, filtered immediately through a sterile 0.22 µm syringe filter, and kept cool for transport to the lab, where they are either analysed immediately or are concentrated by freeze-drying and stored at -20 °C for later analyses.

Basic analyses include the measurement of the concentration of the dissolved organic C in the trap solution using a total organic carbon (TOC) analyser and weighing of the dried root system. Net mass-specific exudation rates (gross root exudation minus reabsorption and microbial consumption) are then calculated as the total amount of C flushed from each root system over the incubation period divided by the total root mass and incubation time (mmol C g^{-1} h^{-1}). Advanced analyses include the identification of the exuded metabolites by ecometabolomics and the morphological and architectural analyses of the root system by optical surface area measurement. While the cuvette method allows the collection and compositional analysis of root exudates from mature trees in their natural environment and therefore is the method that is the closest to investigating the natural exudate composition and exudation patterns

of mature trees, it has to be kept in mind that they may still differ from their original pattern since root exudates are sampled in a medium that differs from a natural soil environment (e.g. with respect to nutrient availability, aeration, and the interaction with soil microbes).

Measurement unit: hours; Measurement scale: length, mass or surface unit of root; ground unit of a plot or site; Equipment costs: €; Running costs: €-€€€ (according to the chemical analysis); Installation effort: high; Maintenance effort: high (frequent cleaning and long exposure time); Knowledge need: medium (species knowledge at the root level); Measurement mode: manual

Sampling with filter papers or agar sheets. The collection of specific exuded compounds can be conducted by the application of filter papers, agar sheets, or resin foils to excised root systems. Similar to the culture-based cuvette method (see the description of the *Culture-based cuvette method* above), the sampled fine root systems are carefully excavated from the soil and remain attached to the plant during the whole sampling process. After excavation, the species identity of the root system has to be confirmed with a morphological key (or by tracking the root system back to the parent tree). **Filter paper** with high soaking capacity is cut into an appropriate size, washed with methanol and distilled water to remove impurities, and applied to the root zones of interest for short time periods (1–3 h; Luster & Finlay, 2006). During this time, the rest of the root system has to be covered with moist filter paper soaked in nutrient solution to avoid drying. The LMWO compounds absorbed by the filter paper (or phenolic compounds by cellulose acetate filters, enzymes by blotting membranes) are re-extracted with water or buffer (according to subsequent analyses), the solution is centrifuged, and the supernatant used for chemical compound or enzymatic analyses. Alternatively, the filter paper can be soaked with artificial substrates for enzymes, which are visualised after colour reaction (Figure 2.1.5.1).

Agar gels have been used as a carrier matrix for Al-aluminium complexes that visualise the exudation of Al-complexing chelators (Luster & Finlay, 2006). The gel sheets are produced in small thicknesses and are applied until discoloration becomes visible. Similarly, the exudation of reducing exudates (e.g. malate, phenolics) for the reduction of Fe-III-oxides can be visualised by the application of agar gels containing Fe redox indicators. Common to all three methods, i.e. sampling with filter papers, agar sheets, or resin foils, is that they allow the analysis of spatial variation of exudation rates along the root axis, but are restricted to specific compounds or compound classes, which are bound by the media, and to small sampling volumes. Consequently, these methods allow only qualitative or semi-quantitative comparison of compounds (e.g. Shi et al., 2011).

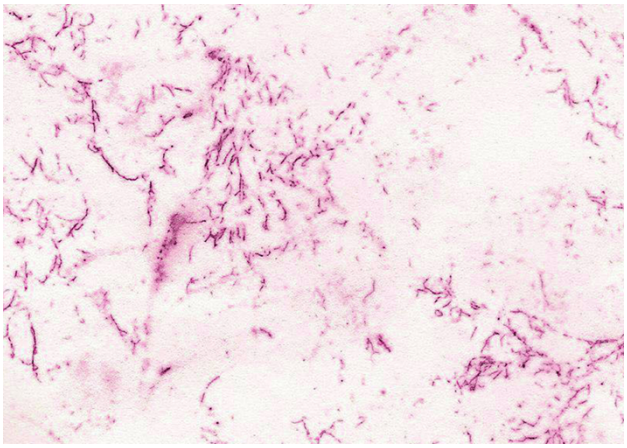

Figure 2.1.5.1 Sampling of acid phosphatase activity with filter paper (photo credit: Ina C. Meier).

Measurement unit: hours; Measurement scale: ground unit of a plot or site; Equipment cost: €; Running costs: €; Installation effort: high; Maintenance effort: low; Knowledge need: medium (species knowledge at the root level); Measurement mode: manual

b) Rhizosoil solution sampling

Micro-suction cups. Rhizosoil solution – root exudation minus re-absorption, microbial consumption, and soil matrix adsorption – can be collected with the help of micro-suction cups. This technique allows non-destructive and repeated *in situ* collection of rhizosoil pore water sampling at high spatial resolution (scale µL mm^{-1}) (Puschenreiter et al., 2005; Dessureault-Rompré et al., 2006). To access the root systems in their undisturbed soil environment an even, flat soil surface has to be experimentally manufactured (e.g. by the installation of soil observation windows or rhizoboxes) a sufficient time period before the collection campaign to ensure new root ingrowth. According to the dynamic of the investigated root systems, installation of these root windows may require one or several years lead time before the actual solution collection can take place. Each micro-suction cup consists of a small diameter, porous tip (with a pore size <0.2 µm that excludes microbial invasion into the samplers) and a tube with a luer lock to connect to vacuum tubes or syringes. The material of the tips is critical as it may induce material-dependent artefacts, i.e. retention of specific compounds. For the installation of the delicate micro-suction cups, a small hole slightly smaller in diameter than the cup diameter (1–2.5 mm) is drilled close to the root segment of interest and the micro-suction cup is carefully pushed with its porous part into the rhizosoil. Any remaining soil holes or loose soil contact are equalised with local soil or soil slurry. The micro-suction cup is connected to a low-pressure vacuum which is applied either via a vacuum pump or a syringe (Figure 2.1.5.2). The retrieved sampling volume is typically very small and the yield of several micro-suction cups may have to be combined for a sufficient volume for later analysis.

Figure 2.1.5.2 Micro-suction cups installed in a rhizobox (photo credit: Ina C. Meier).

For a temporal resolution, micro-suction cups can be repeatedly sampled over a longer time period, but each sampling should alternate with sampling-free equilibration periods, since the sampling of rhizosoil solution imposes changes to the soil moisture and chemical conditions in the rhizosoil (Vetterlein & Jahn, 2004). For high spatial resolution, micro-suction cups are located in close, but different distances

to the root segment of interest or along the root. In extremely dry or clay-rich soil environments, irrigation of the soil from the top can be required to ensure sufficient soil solution for the extraction, but dilutes the rhizosoil solution. The extracted rhizosoil solution can be analysed for dissolved organic C, dissolved gases, and for its elemental composition. A major disadvantage of this method is that both adsorption to the soil matrix and microbial degradation change the exudate composition and concentration and thus quantification of root exudation is not possible.

Measurement unit: days; Measurement scale: plot; Equipment costs: €€; Running costs: €; Installation effort: high; Maintenance effort: high (frequent recording); Knowledge need: medium (species knowledge at the root level); Measurement mode: manual

Micro-dialysis. Micro-dialysis has been introduced as a novel method to sample diffusive fluxes of metals (Miró et al., 2010) and N species from soil (Inselsbacher et al., 2011; Shaw et al., 2014; Brackin et al., 2017). Micro-dialysis is a membrane-based technique for monitoring freely available nutrients and ions in the soil at the root scale. The method is based on the passive diffusion of solutes from rhizosoil across a hydrophilic semipermeable membrane at the end of the probe into a sample collector, while remaining sterile and without the breakdown by microbes or enzymes, which are excluded by the small aperture size of the probes (20 kDa). This means that organic forms of nitrogen (such as amino acids), for example, remain intact (Inselsbacher et al., 2011; Brackin et al., 2015). The micro-dialysis probe is connected to the (syringe) pumping system that pushes water (or any other perfusion liquid (see Miró et al., 2010) through the tubing to the probes in the soil and onto safe-lock vials within the fraction collector (Figure 2.1.5.3; Brackin et al., 2017).

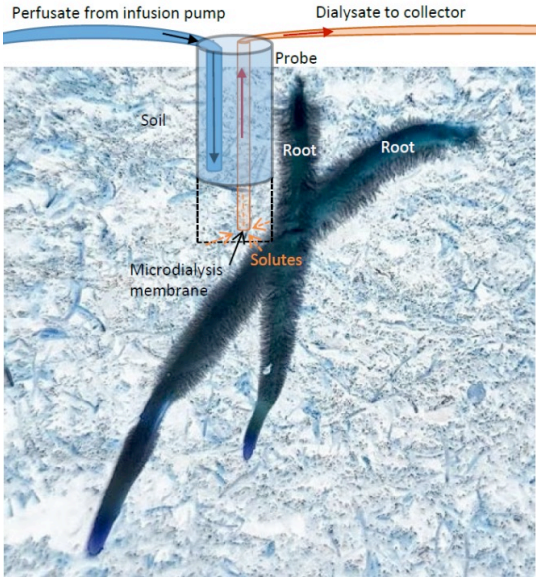

Figure 2.1.5.3 Illustration of the micro-dialysis system, modified from Brackin et al. (2017).

Further details of a probe construction and perfusate flow rate delivery are available in Miró & Frenzel (2004). The miniature design of the probes enables installation with minimal disturbance and high spatial resolution. Micro-dialysis can be used repeatedly over time, removes only dissolved compounds but not soil water, and preserves nutrients in their native form (Miró & Frenzel, 2005; Inselsbacher et al., 2011). Even though accurate calibration and deduction of external soil solution concentrations is problematic (Shaw et al., 2014), the micro-dialysis method avoids biases common to the suction-cup method, such as adsorption to soil particles and dependence on soil pore sizes.

Measurement unit: minutes to hours; Measurement scale: ground unit of a plot or site; Equipment costs: €€; Running costs: €-€€€ (according to the chemical analysis); Installation effort: high; Maintenance effort: high (frequent recording); Knowledge need: medium (species knowledge at the root level); Measurement mode: manual

c) Plant–microbe Interactions

Stable isotope enrichment. Isotope labelling with ^{13}C or ^{14}C is used to trace the partitioning and fate of assimilated C in plants and soil. This method estimates total rhizodeposition, i.e. the release of root cap and border cells, insoluble mucilage, soluble root exudates, volatile organic C, the C flux to mycorrhizae, and the death and lysis of root cells (Jones et al., 2009). For *in situ* isotope labelling, safety regulations restrict the use of C isotopes to the stable ^{13}C. Labelling can be conducted as a pulse, which can be repeated several times during the growing season (Whipps, 1990; Kuzyakov, 2001), or can be conducted continuously, which is more expensive, but results in constant isotopic ratios in all metabolites. The latter is more appropriate for the estimation of the total amount of C transferred from plants to soil (Ge et al., 2015). The separation between root-derived C and soil organic matter C is necessary and can be achieved by labelling of shoots in a $^{13}CO_2$ atmosphere. Isolation of the headspace from the atmosphere has to be achieved, for example by an airtight Plexiglas chamber (Kuzyakov et al., 1999, 2001), and root and shoot zones have to be separated, for example by low melting paraffin overlaid by silicon paste. A sufficient amount of ^{13}C is injected as 99 atom-% ^{13}C-CO_2 into the Plexiglas chamber, while tracking the absolute CO_2 concentration in the chamber. The amount of label needed can be estimated from the strength of the label, the daily assimilation rate of the investigated plant species, and the targeted bulk soil enrichment.

Labelling can take place for short (1–3 h, pulse labelling), repeated, or long time periods (continuous labelling) according to the research question. During the labelling, i.e. the closure of the hoods, the climatic conditions inside the chamber have to be controlled. Subsequently, the remaining unassimilated $^{13}CO_2$ from the Plexiglas chamber is continuously pumped through 1 M NaOH solution as a trap for CO_2 and its concentration in the solution is subsequently analysed. The isotope C ratio in shoots, roots, microorganisms, dissolved organic carbon (DOC), soil, and trap solution are identified by isotope ratio mass spectrometry. The total assimilated ^{13}C is the percentage of ^{13}C added to plants after subtracting the unassimilated ^{13}C. The net assimilated ^{13}C is calculated from the percentage of ^{13}C recovery, i.e. the sum of the ^{13}C in shoots, roots, soil, and in soil respiration. The difference in $\delta^{13}C$ before and after the experiment can be compared with the natural $\delta^{13}C$ signature of roots for an estimation of rhizodeposition and for analysing the partitioning between plants, soil, and microbes. However, plant discrimination against ^{13}C has to be considered for an interpretation of the results.

Alternatively, the natural abundance technique uses the isotopic signature difference between C_3 and C_4 plants to estimate the amount of C release by roots to soil: C_4 plants are planted on C_3 soil or *vice versa* (Cheng, 1996; Rochette & Flanagan, 1998). The method is based on the different isotopic discrimination of $^{13}CO_2$ by Rubisco (ribulose-biphosphate carboxylase) enzymes of C_3 plants and PEP (phosphoenolpyruvate) carboxylase of C_4 plants, which results in a $\delta^{13}C$ signature of c. 27 ‰ in C_3 plants and of c. 14 ‰ in C_4 plants (Tieszen & Boutton, 1989), and consequently also in significant differences in the respective soils. The method is dependent on significant changes of the $\delta^{13}C$ signature in soil and is therefore often applied over one or even several growing seasons for a strong signal and combined with fractionation procedures. Finally, it is also possible to couple natural abundance and ^{13}C pulse labelling for specific research questions (Werth & Kuzyakov, 2006).

Measurement unit: hours to days; Measurement scale: ground unit of a plot or site; Equipment costs: €€-€€€; Running costs: €€-€€€; Installation effort: high; Maintenance effort: medium; Knowledge need: high; Measurement mode: manual

Where to start

Kuzyakov & Domanski (2000), Luster & Finlay (2006), Neumann & Römheld (2007), Oburger & Schmidt (2016), Oburger & Jones (2018) Vranova et al. (2013).

2.1.5.2 Special cases, emerging issues, and challenges

Trap solution medium. Root exudation is influenced by the concentration gradient between roots and soil solution. Consequently, the nature of the trap solution will influence the amount of root exudation and the concentration of specific compounds (Kuijken et al., 2015). Sampling of root exudates in water for short time periods may lead to a potential overestimation of exudation rates as a consequence of osmosis between root cells and the low ionic strength solution (Neumann & Römheld, 2007; Vranova et al., 2013), while sampling in water over a long time period (> 24 h) may lead to an underestimation of exudation rates due to low nutrient availability in the solution (Jones & Darrah, 1993). It is assumed that exudation efflux generally increases and influx decreases with the strength of the solution (Vranova et al., 2013). Ideally, the trap solution should resemble the soil solution as closely as possible, for example by the use of a dilute nutrient solution (pH adjusted) that does not interfere with subsequent chemical analyses. Occasionally, ≥ 100 µM Ca^{2+} is added to the trap solution to ensure root membrane integrity (Vranova et al., 2013). Sterile conditions in the trap solution are sometimes maintained by the addition of a sterilising or protecting agent (e.g. antimicrobial compounds, K_2SO_4), yet rhizotoxic effects on the plant and the efficiency of their force in soil are disputed. Some authors note a negligible effect of microbial degradation for sampling periods of less than 24 h (Jones & Darrah, 1993), while others find significant effects (Kraffczyk et al., 1984).

Incubation period. Time is a very critical factor when sampling root exudates in steady-state *in situ* conditions. The amount of exuded compounds will increase over time and higher concentrations facilitate the chemical analyses, but, at the same time, the number of microbes invading the sampling agent will also increase over time, which decreases the concentration of exudates. Ideally, the exposition time is just long enough to yield enough signal for later analyses (which is species- and site-specific), but short enough to avoid significant microbial consumption (see also review by Oburger & Jones, 2018). The ideal time length should be estimated from prior time series of root exudate collection in combination with the intended chemical analysis. In addition, the diurnal cycle of photosynthate production has to be considered when planning the time for root exudate collections.

Resorption. Potential exudate resorption of the exuded compounds by the same roots has to be considered. C loss to the rhizosphere is generally not a unidirectional flux: a major part of the lost exudate C can be actively retrieved depending on the plant photosynthetic metabolism (Jones & Darrah,

1993; Sacchi et al., 2000). C_4 plants retrieve up to 10% of LMWO compounds; whereas C_3 plants may retrieve up to 70% of the exuded LMWO compounds (see review by Vranova et al., 2013).

2.1.5.3 References

Theory, significance, and large datasets

Neumann & Römheld (2007), Smit et al. (2000).

More on methods and existing protocols

Bromand et al. (2001), Dessureault-Rompré et al. (2006), Kuzyakov (2001), Phillips et al. (2008), Puschenreiter et al. (2005), Wenzel et al. (2001).

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2.1.6 Foliar nutrient stoichiometry and resorption

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Measurement unit: mg g^{-1} or%; Measurement scale: plot; Equipment costs: €; Running costs: €-€€; Installation effort: low to medium; Maintenance effort: -; Knowledge need: low; Measurement mode: manual

Nitrogen (N) and phosphorus (P) are the most widely measured foliar nutrients because they are essential to leaf functions such as photosynthesis and because either or both may limit primary production in terrestrial ecosystems (Elser et al., 2007). Other elements (such as potassium and micronutrients) may also be of interest in specific situations where they are thought to be limiting (Fay et al., 2015; Sardans & Peñuelas, 2015; Kaspari & Powers, 2016). Controls over N and P concentrations and stoichiometric ratios in foliar tissue are complex, but may include environmental factors such as climate, soil type, and nutrient availability, as well as phylogeny, functional type, competition, or interactions with herbivores and parasites (Elser et al., 2010; Rivas-Ubach et al., 2012; Stock & Verboom, 2012; Borer et al., 2015). Stoichiometric ratios have been interpreted as one indicator of relative N or P limitation (Tessier & Raynal, 2003) although they should be interpreted cautiously as findings do not always agree with other limitation metrics such as fertilisation responses (Craine et al., 2008). Foliar stoichiometry and nutrient resorption are of interest in climate-change experiments or observations because they integrate plant metabolic responses to stress. Drought and warming, for example, have been shown to decrease N resorption in temperate trees, while elevated CO_2 typically increases foliar C:N and C:P ratios (Sardans et al., 2012; Suseela & Tharayil, 2017). These metrics can also capture changes in ecosystem nutrient supply that result from climate-driven shifts in microbial nutrient cycling or from other other global-change impacts such as N deposition (which can decrease C:N ratios) (Elser et al., 2010; Suseela & Tharayil, 2017).

Perennial plants typically resorb N, P, and other nutrients from leaves prior to abscission, in order to store and recycle nutrients for the construction of future tissues. Thus, the degree of resorption is an important determinant of total plant nutrient availability (and therefore growth, photosynthesis, etc.), nutrient-use efficiency, and fluxes of nutrients between live biomass and necromass (Reed et al., 2012; Brant & Chen, 2015). These resorption values have been identified as an important parameter in ecosystem and biogeochemical models (Vergutz et al., 2012). Because resorption affects the quality of plant litter (e.g. C:N, C:P, or N:P ratios), it may also influence rates of litter decomposition and thus ecosystem nutrient cycling more broadly (Manzoni et al., 2008; Mooshammer et al., 2012; Suseela & Tharayil, 2017).

Globally, P-resorption efficiency has been shown to be higher than N-resorption efficiency (global leaf N:P ratio = 28:1, global litter N:P ratio = 45:1) but can be highly variable (Zechmeister-Bolstenstern et al., 2015). The degree of resorption can be influenced by factors that also drive stoichiometry such as species, functional type, climate, and external nutrient availability, as well as by initial nutrient status and stresses including early frost or storms that cause leaf damage or premature abscission (Killingbeck,

1988; Reed et al., 2012; Xu et al., 2017). Additionally, the degree of resorption may also be cautiously interpreted as an indicator of relative nutrient limitation (Killingbeck, 1996; Reed et al., 2012; Vergutz et al., 2012, Zechmeister-Bolstenstern et al., 2015), although care should be taken to account for potential interactions between elements (See et al., 2015).

2.1.6.1 What and how to measure?

Sampling of live foliage (nutrient concentrations) or litter (resorption) is the most common measurement approach, although remote sensing may also be appropriate for estimating some foliar nutrients at broader scales. Manual sampling is straightforward and relatively low cost (depending on sampling intensity, canopy accessibility, and analysis method). Although destructive, this method requires relatively little tissue (in the order of a few grams dry material), most of which is used to generate a homogenous sample, rather than required for analysis.

The difficulty of sampling foliage is dependent on plant growth form. For vegetation up to several metres in height, full sun leaves can generally be reached directly or with the aid of a pole pruner. Beyond that, tall trees can often be sampled by shotgun or slingshot, but otherwise may require technical tree climbing skills and equipment.

Sampling requires collection of fully expanded, healthy, green, sun-lit and shade leaves. When sun and shade leaves are analysed separately, leaf nutrient concentration is taken as the weighted mean considering the proportion of each leaf type. Because inter-specific variation can be large (Townsend et al., 2007; Reed et al., 2012), individual species should be considered separately rather than pooled. In seasonal ecosystems, the best time to sample is at the height of the growing season, when foliar N has stabilised (but see Zhang et al., 2013) but before any signs of senescence have begun. It is preferable to sample multiple leaves from each plant (e.g. in each cardinal direction) and either combine them to form a homogenous sample or to analyse them separately to quantify variability. As foliar N and P can vary with leaf age in evergreens (Mediavilla & Escudero, 2003), care should be taken to collect a representative sample of the whole plant if individual leaf cohorts cannot be distinguished. The decision of whether to include petioles with leaves in analysis is discussed in Pérez-Harguindeguy et al. (2013).

Resorption measurements necessitate returning to resample the same individuals (or the same species within the same area) by collecting recently dropped, fully senesced leaves. To avoid bias, it is preferable that the same individual repeats sampling. Fallen leaves can be collected with litter traps or tarps, or by shaking from the plant, and should preferably be collected soon after abscission to avoid leaching of nutrients and decomposition that can begin quickly, especially when wet. In mixed species stands, it may be necessary to identify and sort litter by species.

Leaves should preferably be sampled into paper, rather than plastic, bags unless they will be dried immediately. Samples can be air dried if necessary, and ultimately placed in a drying oven at a maximum of 70 °C for 72h until they have reached constant weight (Pérez-Harguindeguy et al., 2013). The temperature and time for drying depend on the study question, how many samples are dried, the size, thickness and type of the plant material (e.g. large, fleshy or succulent leaves need more time) see protocol 2.1.1 Aboveground plant biomass for more details on the drying. Tissue is then ground to a fine

powder using a ball or Wiley Mill, a mortar and pestle and liquid N, or an electric coffee grinder. The latter is an economical solution that can work well for moderately tough leaves.

To generate stoichiometric ratios (e.g. C:N or N:P) it may be necessary to employ more than one analytical method. Sample nutrient concentrations can be measured in a variety of ways, an overview of which can be found in van Heerwaarden et al. (2003). Generally, colorimetric methods have a lower accuracy and a higher detection limit than elemental analysers, but are more cost effective and require less specialised analytical equipment. Elemental analysis services are commonly provided by commercial or university service lab facilities. If isotopic analysis is performed on foliar samples, C and N content can be derived from these.

Foliar nutrient concentrations can be expressed either as a percent value (%) on a dry mass basis or in units of mg g^{-1} dry mass. For needle leaves, nutrient concentrations are commonly expressed per unit area rather than mass (Smith et al., 1981). Resorption is usually calculated as *efficiency* (percent of initial nutrients resorbed; high value = more efficient resorption), but reference can also be made to *proficiency* (concentration of nutrients left in sensed tissue; low value = more proficient resorption; Killingbeck, 1996).

Resorption efficiency, calculated as percent C or N content in leaves (Killingbeck, 1996):

[live] - [senesced] / [live] * 100

Resorption values can also be expressed per unit leaf area, or can be corrected to account for mass loss during senescence (van Heerwaarden et al., 2003; Vergutz et al., 2012). To correct for leaf mass loss the shrinkage in LMA between green leaves and leaf litter must be known.

There is a large body of literature on both foliar nutrient concentrations and resorption ratios available to contextualise results across many ecosystem types (e.g. Killingbeck, 1996; Townsend et al., 2007; Reed et al., 2012; Vergutz et al., 2012; Xu et al., 2017).

Where to start

Brant & Chen (2015), Elser et al. (2010), Killingbeck (1996), Vergutz et al. (2012), Zechmeister-Boltenstern et al. (2015)

2.1.6.2 Special cases, emerging issues, and challenges

Loss of tissue prior to senescence

Foliar tissue can be lost from plants prior to the completion of normal senescence and nutrient resorption, often as a result of stresses such as frost or hurricane damage (Killingbeck, 1988) or drought (Estiarte & Peñuelas, 2015). This process would tend to reduce nutrient resorption and can be quantified as described above if suitable samples can be obtained.

Grasses

As perennial grasses might not obviously drop senesced leaves, resorption can be measured by tagging fully expanded live leaves and monitoring them until they are considered thoroughly senesced by turning yellow or brown (e.g. Lü et al., 2011).

Nutrients in other plant tissues

Nutrient concentrations and resorption may also be of interest in other tissues with high turnover rates, particularly fine roots. While the protocol is essentially the same, designing root sampling is more challenging and requires additional considerations including the definition of fine roots, potential mass loss, the ability to separate roots of co-occurring species, and the ability to distinguish senescence (Kunkle et al., 2009; McCormack et al., 2015).

Remotely-sensed foliar nutrients

Airborne imaging spectroscopy by high spatial resolution (< 5 m) platforms mounted on drones or airplanes is increasingly used to measure foliar nutrients, most notably N (Asner & Martin, 2008; Lepine et al., 2016; also see protocol 5.12 Reflectance assessment of plant physiological status). Although these measures are generally applied at the plot or landscape scale, individual species and crowns can be identified from remotely sensed data with increasing accuracy (e.g. Chadwick & Asner, 2016). Platforms that measure foliar N can often concurrently provide some information on a suite of other chemical traits such as lignin or cellulose content (Martin & Aber, 1997; Asner et al., 2015).

2.1.6.3 References

Theory, significance, and large datasets

Brant & Chen (2015), Reed et al. (2012), Townsend et al. (2007), Vergutz et al. (2012), Xu et al. (2017)

More on methods and existing protocols

Pérez-Harguindeguy et al. (2013), van Heerwaarden et al. (2003)

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2.2 Soil

2.2.1. Soil microbial biomass - C, N, and P

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Measurement unit: mg C, N, or P g^{-1} dry soil or g^{-1} SOM; Measurement scale: plot; Equipment costs: none; Running costs: €; Installation effort: low; Maintenance effort: medium; Knowledge: medium; Measurement mode: manual

The soil microbial biomass constitutes 1–3% of the total soil organic carbon (C) content. Soil microbes also store a substantial amount of nutrients, nitrogen (N), and phosphorus (P) as the C:N and C:P ratios in microbes are relatively low compared to plant nutrient ratios (Schmidt et al., 2002; Zechmeister-Boltenstern et al., 2015). For example, microbial biomass P was about one-third of total soil P in an arctic heath (Jonasson et al., 1996, 1999), and on a global scale, estimates suggest that soil microbial N and P pools are equal to plant nutrient pools – despite plants storing far more C relative to microbes (Whitman et al., 1998; Bar-On et. al., 2018). Hence, even small fluctuations in microbial biomass may be crucial for mobilisation (release) and immobilisation (microbial uptake) rates of N and P in nutrient-deficient soils, i.e. where N and/or P limit plant growth (Smith & Paul, 1990; Schmidt et al., 2002). Consequently, microbial biomass pools are highly important components of an ecosystem's carbon and nutrient storage potential and the pools are important to take into account when evaluating responses to experimental climate change or other drivers of global change.

2.2.1.1 What and how to measure?

Gold standard

Microbial biomass in soil can be quantified by the chloroform-fumigation extraction (CFE; Brookes et al., 1985b, also see Table 2.2.1.1). First, roots and stones (> 2 mm diameter) are removed from the fresh, homogenised soil samples. Then, one set of soil samples (minimum 10 g) is extracted for soluble C (dissolved organic carbon; DOC), N (total dissolved nitrogen; TDN), and P (PO_4). Sorting out roots by hand is preferred as sieving may release substantial amounts of nutrients and if the sorted soil is stored, the microbes may utilise the released C and nutrients.

Simultaneously, another set of soil samples (minimum 10 g) are vacuum-incubated and treated with ethanol-free chloroform fumes for 24 h at room temperature (~ 20 °C) in the dark (Jenkinson & Powlson, 1976; Tate et al., 1988). This fumigation step is performed using a desiccator and a pump placed in a well-aerated fume-hood. The chloroform treatment kills and lyses the living soil microorganisms (Brookes et al., 1985a, 1985b), releasing microbial C, N, and P contents into the soil solution (Vance et al., 1987). As chloroform is not only toxic for microbes, special care has to be taken when handling the chloroform as well as chloroform-treated soils. After 24 hours, the fumigated soil samples are extracted similarly to the non-fumigated samples. Microbial C, N, and P are commonly reported in mg C, N, and P

per grams of dry soil or per grams of soil organic matter (SOM) content. Thus, a third soil subsample is needed for fresh to dry weight conversion – followed by loss on ignition combustion if SOM content is desired.

Microbial biomass C, N, and P is calculated as the difference in C, N, or P contents in pair-wise extracts of unfumigated and fumigated soil samples, for example:

$$\text{Microbial biomass C (mg C per g dry soil)} = C_{\text{fumigated soil}} - C_{\text{unfumigated soil}}$$

Table 2.2.1.1 Stepwise guide to microbial biomass measurements. The first row describes how soil samples are processed in the laboratory prior to fumigation. The second row is a guide to chloroform fumigation of soil samples. Another set of soil samples are extracted for initial content of inorganic and dissolved organic nutrients (third row), followed by a procedure to stop fumigation (fourth row). Rows five and six describe subsequent analytical analyses of the fumigated and unfumigated extractants, and the necessary calculations, respectively.

	Step 1	**Step 2**	**Step 3**	**Step 4**
Soil samples	Sort out roots by hand	Weigh three sets of samples for an un-fumigated sample, a fumigated sample and for fresh:dry weight conversion	Place the fresh soil in an oven and dry at e.g. 55 °C until constant weight	Weigh the dry soil and calculate water content so all measures can be expressed on a g dry weight soil basis
Start fumigation	Use aluminium containers (alu-candle holder) or crucibles as chloroform will dissolve plastic	Place soil samples in dessicator – line bottom of desiccator with wet napkins to avoid soil drying	Clean chloroform for stabilising ethanol. Pass 10–30 ml through Al_2O_3 into a beaker. USE FUME HOOD!	Place $ChCl_3$ beaker in dessicator and close the lid. Start the vacuum pump and continue until chloroform boils. Close the valve AND then turn off the pump.
Unfumigated sample	Extract soil sample in appropriate extractant (see details below) when the fumigated soil samples are in the dessicator	Filter and store cold. Samples can be stored in the freezer if more than a few days will pass before analysis, which should be at the same time as the batch of fumigated samples		
Stop fumigation	Open the valve of the desiccator after 24 h and let air enter very carefully	Open the desiccator and remove the wet napkins and the $ChCl_3$ beaker with the remaining chloroform	Close the lid on the desiccator and flush repeatedly 3–4 times, using the pump to remove remaining chloroform fumes	Remove the dessicator lid once more and take out the fumigated soil samples. These are now ready for extraction – same procedure for the unfumigated samples

Laboratory analyses	Measurement of DOC in extracts of fumigated and unfumigated samples for microbial C assessment	Measurement of TDN in extracts of fumigated and unfumigated samples for assessment of microbial N	Measurement of PO_4-P in extracts of fumigated and unfumigated samples for assessment of microbial P	
Calculation	Microbial biomass C, N, and P are calculated as the difference in C, N, or P contents in pair-wise extracts of unfumigated and fumigated soil samples			Note that all extractions must be corrected for the soil sample specific dilution associated with its gravimetric soil moisture content.

According to the literature, fumigated and unfumigated soil samples have most often been extracted with 0.5 M K_2SO_4, which is suitable for obtaining C and N. However, using a lower concentration of K_2SO_4 (0.05 M) or even extraction in pure water are highly recommended alternatives (see *Special cases* for details; Nordin et al., 2004). The extraction is followed by measurements of DOC, inorganic N, TDN, and PO_4 on extracts of both fumigated and non-fumigated samples.

In dry soils (with a water content below 30% of field-capacity), the water content can be adjusted by adding a known amount of pure water to the samples right before the on-set of fumigation. Alternatively, when dealing with waterlogged soils, a modified chloroform-addition method has been developed (Witt et al., 2000). Here, chloroform is added directly into each inundated soil sample, and then samples are incubated for 24 h and extracted as described above.

Handling of soil samples for microbial C, N and P content

Microbial biomass C, N and P are usually measured in the top 10 or 30 cm of the soil where the microbial biomass and activity are highest. In the field, soil samples are placed in a cooling box at ca. 4 °C in a cooling room back in the laboratory. The samples should NOT be frozen or dried. The samples are stored as intact soil cores to minimize disturbance and microbial activity. The microorganisms are active also at low temperatures, so the samples should be processed within few days after sampling in the field following the steps in Table 2.2.1.1.

Where to start

THE CFE method has generally not changed much over the past 30 years, and details on the procedure are found in the classic method papers by Brookes et al. (1985b), Vance et al. (1987), and Tate et al.

(1988). For current studies using the CFE method with slight modifications, see, for example, Christiansen et al. (2018).

2.2.1.2 Special cases, emerging issues, and challenges

Use of extractants

Historically, K_2SO_4 has been the preferred extractant used for microbial C and N extractions. However, 0.5 M K_2SO_4 is difficult to work with because i) the solution concentration is close to saturation, ii) possible salt precipitation occurs within sample vials when cooled or frozen, and iii) samples often leave salt deposits inside the instruments during analysis. Similar biomass pool sizes can be obtained when using low concentration 0.05 M K_2SO_4 or simple H_2O extraction (Nordin et al., 2004). A test of extractability with 0.05 M K_2SO_4 or H_2O shows no effect on microbial C, but the extractability of inorganic and dissolved organic N is approximately 20% lower with 0.05 M K_2SO_4 and 30% lower with water relative to 0.5 M K_2SO_4 (pers. obs.). If desired, this extractability difference can be adjusted for by using a lower K_{EN} factor of 0.45 compared to 0.54 used by Brookes et al. (1985b), but see recommendations on extraction factors below.

$NaHCO_3$ is generally used to extract PO_4-P and microbial P in neutral to alkaline soils (Schmidt et al., 1999), while Bray-1 solution is used for PO_4-P and microbial P in acidic soils (Bray & Kurtz, 1945; Wu et al., 2000); see Zederer et al. (2017) for details on microbial P determination.

Using extraction correction factors

Extractability of microbial C, N, and P varies across different soils (Joergensen, 1996; Joergensen & Mueller, 1996), microbial communities (Anderson & Domsch, 1978), and seasons (Schadt et al., 2003). CFE extraction efficiency is generally 40–60% of total microbial biomass, and extraction correction factors K_{EC}, K_{EN}, and K_{EP} are often applied to adjust for the incomplete release of C, N, or P, respectively (Jonasson et al., 1996), for example, when absolute pool sizes are desired, such as when calculating total ecosystem pools or budgets. However, great care should be taken to apply a suitable correction factor – and it is relatively common not to use any correction factor at all. Based on the literature, general correction factors for microbial biomass C, N, and P are K_{EC} = 0.45 (Wu et al., 1990; Joergensen, 1996), K_{EN} = 0.54 (Brookes et al., 1985a), and K_{EP} = 0.40 (Jonasson et al., 1996), respectively. Always specify whether correction factors were used in the calculations and what values were applied as correction.

Fatty acid analyses

The CFE method measures the total microbial biomass, i.e. combined bacterial and fungal C, N, and P. An ergosterol assay has been developed to quantify the fungal abundance in soil (Salmanowicz & Nylund, 1988; Nylund & Wallander, 1992). In contrast, fatty acid methyl esters (FAMEs) extracted from microbial cell walls are used to quantify the relative bacterial (PLFAs – phospholipid fatty acids) and fungal (mainly NLFAs – neutral-lipid fatty acids) abundance (Bligh & Dyer, 1959; Frostegård & Bååth,

1996). FAMEs are also used to broadly describe the microbial community composition. More advanced methods use sequences of genetic material (e.g. 16S and ITS regions of rRNA or DNA) of bacteria or fungi, respectively, to identify microbial community composition at a finer scale (Marsh, 1999), and qPCR can be used to quantify cell numbers which can then be used for biomass estimations (see 4.9 Soil microbial community composition). A comprehensive review of methods studying soil microbial diversity is published by Kirk et al. (2004). Note that there is generally a good agreement between biomass estimations based on CFE and PLFA methods (Schmidt et al., 2000).

CFE modifications relating to soil type

In dry soils with a water content below 30% of field capacity, the water content can be adjusted by adding a known amount of pure water to the samples immediately before the on-set of fumigation. Alternatively, when dealing with waterlogged soils, a modified chloroform-addition method has been developed (Witt et al., 2000). Here, liquid chloroform is added directly into each inundated soil sample, and then samples are incubated for 24 hours before extraction. For soils that are high in clay content, note that chloroform fumes can adsorb to clay minerals and potentially bias the microbial biomass C measurement – see Alessi et al. (2011) for uncertainties in biomass C estimates with the CFE.

Isotopic signatures of microbial C and N

Microbial C and N can be quantified using stable C and N isotopes. Stable isotope analysis allows for the quantification of C and N use by microbes under different climatic conditions in stable isotope labelling experiments (Nordin et al., 2004; Andresen et al., 2009, 2018; Reinsch et al., 2014) or continuous labelling experiments e.g. Free-Air CO_2 Enrichment (FACE). Stable isotope analysis of soil extracts can also be used when C and N stabilisation in the microbial biomass is of interest (Thaysen et al., 2017). Isotopic values of soil microbial C and N can be determined after the CFE method. Unfumigated and fumigated soil samples are extracted in water (for later analyses of DOC and TDN) and then freeze-dried (Lipson & Monson, 1998). Extraction of soils with K_2SO_4 will leave salt deposits after freeze-drying and make packaging for stable isotope analysis difficult; K_2SO_4 deposits further ruin the separation columns used in isotope ratio mass spectrometry even at low salt concentrations. Thus, we recommend the use of water extraction so that solvent from the samples can be evaporated during freeze-drying without leaving a salty residue during stable isotope analysis of $\delta^{13}C$ and $\delta^{15}N$ (Nordin et al., 2004).

Prior to freeze-drying, pH in the extracts should be adjusted in extracts from non-acidic sites to 4.5 with HCl. The soil extracts are sublimated to dryness in a freeze-dryer. $\delta^{13}C$ and ^{15}N concentrations (in atom % compared to isotopic ratios of $^{13}C/^{12}C$ and $^{15}N/^{14}N$) of soil microbes are calculated as the difference of ^{15}N and ^{13}C fumigated and unfumigated samples. However, it is difficult to collect all N and P after freeze drying so the isotopic analysis provides a C:N ratio of the microbial biomass and the excess ^{15}N and ^{13}C. To calculate the pool sizes, the unfumigated and fumigated extracts from the CFE analysis should be analysed for total organic C and TDN to provide the pool sizes.

2.2.1.3 References

Theory, significance, and large datasets and more on methods and existing protocols

The fumigation-extraction method has not changed significantly over the last decades, and details on the procedure are found in the classic method papers by Brookes et al. (1985b), Vance et al. (1987), and Tate et al. (1988). Further, key references will depend on the actual study in question. A key reference for PLFA in microbes is Frostegård & Bååth (1996).

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2.2.2 Root and soil colonisation by mycorrhizal fungi

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Measurement unit: percentage of colonisation; Measurement scale: plot or plant; Equipment costs: €; Running costs: €; Installation effort: medium to high; Maintenance effort: -; Knowledge need: medium to high; Measurement mode: manual

Mycorrhizae are plant–fungi symbiotic relationships in which plants benefit from fungi-derived nutrient supplies and protection from environmental stresses while fungi are provided with plant-synthesised carbon (Smith & Read, 2008). Arbuscular mycorrhizal fungi (AMF), with their namesake nutrient-exchange structures called arbuscules, are the most common type of mycorrhizal fungi, associating with more than 80% of vascular plant species including both herbaceous and woody plants (Bueno et al., 2017). The second most abundant mycorrhizal association is Ectomycorrhiza (EcM) which occurs in 2% of seed plants (Maherali et al., 2016), most of which are highly prevalent temperate tree species. Mycorrhizal fungi can strongly affect plant nutrient uptake, biomass, and photosynthesis as well as the carbon allocation (Bago et al., 2000). Thus, they can play an important role in global carbon and nutrient cycling processes (Hodge et al., 2001; Veresoglou et al., 2012; Soudzilovskaia et al., 2015). In many ecosystems, mycorrhizae, together with fine roots, provide the largest input of carbon into soils (Kramer et al., 2010; Verbruggen et al., 2016). Mycorrhizal symbionts hence determine the flow of vast quantities of carbon on global scales, where the impact of AMF alone may be as large as 4.5 billion tonnes of carbon annually (Bago et al., 2000). The intensity of plant root colonisation by AMF is expressed as a percentage of root length colonised by the fungi (Soudzilovskaia et al., 2015). This is the single best available measure that quantifies the "strength" of the plant–fungi relationship *in situ* and it can indicate the reliance of plants on mycorrhizae in a particular ecosystem (Soudzilovskaia et al., 2015). This is likely positively correlated with nutrient provision by, and C supply to, AMF, but it is not a direct measure, so it should be interpreted with care. AMF can also be isolated directly from the soil which can provide information about the "extraradical" part of AMF consisting of hyphae foraging for nutrients. EcM fungi can be quantified in an analogous process. Quantifying root colonisation by mycorrhizal fungi can be used as a measure of the strength of plant–fungal relationships and an indicator of nutrient and carbon flow between plants and fungi in both manipulation and observational experiments investigating the effect of environmental changes, such as warming (Wilson et al., 2016), elevated CO_2 (Staddon et al., 1999), nitrogen enrichment (Jumpponen et al., 2005), and land-use change (Xiang et al., 2014) on ecosystem functioning.

2.2.2.1 What and how to measure?

Root colonisation

Depending of the study question, either the roots of particular plant species are excavated to assess the mycorrhizal colonisation of these plant species, or, if the colonisation of all roots within a soil sample is

of interest, a soil core is taken (ca. 10 g of soil) from the experimental plot usually at 0-5 or 5-10 cm depth. The samples can be stored in sterile plastic ziplock bags and, if not processed immediately, they should be kept in the freezer (at -20 °C). Typically, between 3 and 5 samples are pooled to represent an experimental unit. Root colonisation varies over time but is highest around the time of flowering, which represents an optimal time for sampling (e.g. Gosling et al., 2013), although earlier development can also be of interest as it is likely relevant for plant development. Subsequently, live-roots are picked out from soil following, for example, the root extraction-flotation method (Cook et al., 1988). Briefly, the soil with roots is washed repeatedly under running water over a sieve (with a mesh size < 1 mm) until the soil particles are completely removed (see also Pérez-Harguindeguy et al., 2013).

After extraction, roots are kept fresh at 4 °C or in 70% ethanol followed by (optional) determination of length of roots according to the method described by Tennant (1975). Alternatively, the roots can be scanned and analysed using dedicated software (for some examples see Cai et al., 2015). In order to measure root length colonisation of AMF, the roots are first cleared in 5–10% KOH in a 90 °C waterbath. Thicker and more coloured roots require more time than small and less coloured roots. Highly pigmented and lignified roots can additionally be bleached or subjected to H_2O_2 to remove any remaining phenolic compounds and pigmentation. In order to make AMF structures visible under the microscope, the roots need to be stained. This is commonly done using Trypan Blue as in Phillips & Hayman (1970) or ink and vinegar as in Vierheilig et al. (1998). Approximately 20 pieces of stained roots (ca. 2 cm long each) are randomly selected and aligned on a microscope slide. The percent of AMF colonisation can then be estimated using the magnified intersections method, described by McGonigle et al. (1990).

In order to avoid biases, it is very important that the selection of root pieces and the location of the cross-hair (see McGonigle et al., 1990 for description) are random. It is also necessary to check the success of root staining, for instance by examining if the stele (vascular tissue) is stained, which is a good indication that the staining is successful. If the whole root is translucent, this indicates that the staining was not efficient and these pieces should not be counted as non-infected. The roots that only have a stele, an incomplete cortex, or those which are heavily colonised by dark-septate fungi (as a signal of potential root mortality) should be excluded from the analysis. Furthermore, given that the method requires recognising AMF hyphae, arbuscules, and vesicles, some training is required, for instance starting with pictures/descriptions at https://mycorrhizas.info/. One distinguishing feature of AMF contrasted against most other fungi is that their hyphae are aseptate, meaning that they are not regularly subdivided by septa (crosswalls within the filaments). The same website can be used to get acquainted with the appearance of EcM fungi. Because these fungi form a sheath around tree roots, fresh roots can be examined submerged in a petri dish under a dissecting microscope and EcM root tips can be expressed as the percentage colonisation of total root tips.

Soil colonisation

The hyphae are extracted from 4 g of soil using the aqueous extraction method as described in Jakobsen et al. (1992). This method involves the use of strong mechanical force (i.e. blending at high speeds) which could cause hyphae to break (thereby possibly hindering the identification of different hyphal

types). To avoid this, the method above can be modified as in Rillig et al. (1999), using sodium hexametaphosphate to break up soil aggregates and bring all hyphae into suspension. The soil suspension is carefully decanted over a 38 µm sieve and gently sprayed to remove clay particles. Then, material left on the sieve is flushed in a known volume of water (e.g. 200 ml) and a subsample is brought on a nitrocellulose filter on a vacuum manifold to isolate the hyphae. The filter is stained with an ink and vinegar solution (or 0.02% Trypan Blue in lactoglycerol) and inspected using a microscope at x200 magnification. Hyphal length can be determined using the visual gridline intersection (VDI) method, which is based on the examination of the frequency of hyphal intersections with gridlines on a microscope eyepiece. The hyphal length is obtained by incorporating the number of hyphae crossing with gridlines into the formula by Newman (1966) (as in, for example, Camenzind & Rillig, 2013). Hyphal extraction efficiency is determined by re-extracting hyphae based on the method by Miller et al. (1995) and this can be used to adjust the values of hyphal lengths accordingly.

Where to start

Camenzind & Rillig (2013), McGonigle et al. (1990), Miller et al. (1995), Rillig et al. (1999), Vierheilig et al. (1998)

2.2.2.2 Special cases, emerging issues, and challenges

Measurement of hyphal length in soil is simple and cost efficient, but rather time-consuming since it requires an experienced eye in distinguishing AMF hyphae from non-AMF hyphae. Alternative methods, based on photomicrography and image processing offer semi-automated analysis of hyphal length where observer biases are minimised (Shen et al., 2016) but do not distinguish AM and non-AM fungi.

To estimate AMF biomass in the roots and soil, the neutral lipid fatty acid (NLFA) 16: 1ω5 (Olsson, 1999) can be used as an AMF specific biomarker (see protocol 2.2.1. Soil microbial biomass - C, N, and P for a small description of PLFA/NLFA analysis). There is no specific biomarker for EcM fungi, although when EcM tree roots are colonised, the majority of fungal PLFA markers or ergosterol will generally originate from EcM fungi.

2.2.2.3 References

Theory, significance, and large datasets

Bago et al. (2000), Bueno et al. (2017), Maherali et al. (2016), Smith & Read (2008), Soudzilovskaia et al. (2015)

More on methods and existing protocols

Cai et al. (2015), Cook et al. (1988), Pérez-Harguindeguy et al. (2013), Phillips & Hayman (1970), Shen et al. (2016), Tennant (1975)

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2.2.3 Soil CO_2 (and other trace gas) fluxes

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Measurement unit: µmol m^{-2} sec^{-1}; Measurement scale: plot; Equipment costs: €€-€€€; Running costs: €-€€; Installation effort: medium to high; Maintenance effort: medium; Knowledge need: medium to high; Measurement mode: manual and/or data logger

Soil CO_2 efflux, often denoted as soil respiration, is one of the largest carbon fluxes in terrestrial ecosystems and therefore regularly assessed in climate-manipulation studies. In vegetated ecosystems, CO_2 efflux from soil comes from two primary sources: heterotrophic (decomposition) and autotrophic (root and rhizosphere) respiration (e.g. Harris & van Bavel, 1957; Högberg et al., 2001). A third CO_2 source can arise during carbonate dissolution/formation. Such abiotic CO_2 fluxes usually play a negligible role (Schindlbacher et al., 2015), but can become significant in arid or semi-arid ecosystems (Serrano-Ortiz et al., 2010).

It is not yet fully resolved how global warming, rising atmospheric CO_2 concentrations, and changing precipitation patterns will affect future soil CO_2 efflux (e.g. Vicca et al., 2014; Romero-Olivares et al., 2017). In particular, the longer-term responses of soil CO_2 efflux to climate change remain unclear (Crowther et al., 2016). Since the major sources of soil CO_2 efflux arise from C pools of different residence time (autotrophic: short lived; heterotrophic: longer lived), it is critical to assess how the different sources are affected by climate change. Assessment of CO_2 efflux in climate manipulation experiments or observational studies can therefore become a complex task. The interaction of changing climate with other global-change phenomena such as nitrogen deposition or land-use change will add even more complexity to the response of soil CO_2 fluxes (Janssens et al. 2010; Sheng et al. 2010).

Besides CO_2, soils can also be important sources or sinks of the greenhouse gases CH_4 and N_2O (Schulze et al., 2009). CH_4 and N_2O measurements can be combined with those of CO_2 (depending on the analyser) and are therefore also integrated into this protocol. The measurement principles of reactive short-lived trace gases such as NO_x are not specifically addressed in this protocol. A description of thorough NO_x flux assessment can be found in, for example, Pape et al. (2009).

2.2.3.1 What and how to measure?

Gold standard

Soil CO_2 efflux is defined as the flow of CO_2 through the soil surface. The common expression is µmol m^{-2} sec^{-1}, or Tonnes C ha^{-1} and kg C m^{-2} when reported on an annual basis. The most commonly used methods for soil CO_2 efflux determination are chamber measurements. Chambers are placed on the soil surface, and air circulates between the closed chamber headspace and a CO_2 detector (closed-dynamic or non-steady state chamber), or the detector is located directly in the chamber headspace. The increase in headspace CO_2 concentration over time allows the soil CO_2 efflux to be inferred. Soil CO_2

efflux can be measured manually with transportable measuring devices (spatial replication) or by using automated systems operating at higher temporal resolution (temporal replication).

Most commonly, infrared gas analysers (IRGA) are used for CO_2 detection. There is an array of different IRGAs and IRGA-chamber combinations for soil CO_2 efflux measurements on the market. An assessment of various systems can be found in Pumpanen et al. (2004), but companies are constantly improving their products. In addition to IRGAs, there is a new generation of (also portable) laser-based technologies entering the market. These instruments enable simultaneous measurements of CO_2, CH_4, and N_2O. On the downside, they are costly and heavy to carry, but the development of new lasers proceeds fast. The possibility to measure these gases alongside CO_2 and at a similar temporal resolution means that similar protocols as described here for CO_2 may be applied when using these gas analysers.

Bronze standard

Gas samples can also be obtained from closed (closed-static) chambers and analysed later on in the laboratory, for example by gas chromatography (GC). Gas samples are repeatedly taken with a syringe and collected in glass vials. This static approach is frequently applied when CH_4 or N_2O fluxes are assessed by GC. In comparison to the dynamic method described above, sample collection is labour-intensive and has a higher potential of bias during vial preparation, sample collection, storage, and transport. Long chamber closing times (often necessary for N_2O / CH_4; see Pihlatie et al., 2013) should be strictly avoided for CO_2 efflux measurements as they result in substantially underestimated soil CO_2 efflux rates. On the positive side, the method offers the opportunity to sample a larger set of chambers simultaneously, thereby saving time and creating a potential for higher spatial replication. Due to the various sources of bias, we only recommend this method when no IRGA or laser is available.

Where to start

Pape et al. (2009); Pihlatie et al. (2013); Pumpanen et al. (2004); Romero-Olivares et al. (2017); Vicca et al. (2014)

Installation, field operation, maintenance, interpretation

The handbook “Soil Carbon Dynamics – An Integrated Methodology” (Kutsch et al., 2009) provides detailed information on the principles of soil CO_2 efflux measurements, the constraints and limitations of different methods, flux-upscale, and experimental design. We highly recommend this book to anybody dealing with CO_2 efflux measurements. Here, we specifically concentrate on practical advice with regard to climate-manipulation experiments.

Manual soil CO_2 efflux measurements (+ cheap, easy to apply, - low temporal coverage-to-workload ratio)

Depending on the studied ecosystem and the specific research question, a system suitable for soil CO_2 efflux measurements has to be chosen according to its size and design. It is up to the researcher to decide whether a commercially available analyser/chamber combination meets their needs, or whether they shall construct their own individual chamber system. The measurement principle of soil CO_2 efflux is relatively simple and homemade chambers can do a good job if they are thoroughly constructed (e.g. larger (diameter > 20–30 cm) chambers should contain an appropriate fan for proper air mixing; a simple vent can avoid over-pressure during chamber closure).

When choosing a chamber, several criteria need to be considered:

- **Colour**: to avoid photosynthetic activity of small plants or algae, chambers for soil CO_2 efflux are typically dark. It is advisable to use bright or reflecting exterior material/colour to avoid unwanted air/soil warming inside (especially in an open field). Under specific climate manipulation treatments, such as infrared warming, the heat conductivity of the collar/ chamber material should be tested and the appropriate material selected in order to avoid potential overheating.
- **Size**: commercially available chambers frequently cover only a relatively small soil surface area (e.g. 10 cm diameter). Under heterogeneous soil conditions, as in many forests, the application of such chambers can result in highly variable measurements of soil CO_2 efflux rates within a short distance. As a result, a correspondingly high number of individual chamber measurements is required to cover spatial heterogeneity to make manipulation treatments comparable. The application of larger diameter chambers (covering more soil and thereby reducing variability) can be helpful in heterogeneous systems. On the other hand, small diameter chambers may fit within narrow bare-soil spaces between or under the plants, thus reducing bias due to the cutting or removal of plants. Chamber height is commonly between 10 and 20 cm to ensure a good balance between being low enough to enable a considerable increase in headspace CO_2 in a few minutes but still high enough to avoid excessive turbulence (created by the fan).
- **Collar**: chambers are typically placed on permanently installed collars or the whole chamber is permanently installed and closed with a lid during measurements. Collar/chamber insertion depth should be as shallow as possible to avoid root cutting (Wang et al., 2005); usually 1–2 cm insertion depth is sufficient to gain a sealing effect between soil and collar/chamber.
- **Chamber air mixing**: sufficient mixing of the chamber atmosphere using a fan, for example, provides a clearer signal of headspace CO_2 over time. However, chamber wind speed should be carefully considered depending on chamber size and design. Too low or too high air mixing may cause boundary layer formation or pumping of air from the soil to the chamber, respectively, both leading to biased flux estimates (Hooper et al., 2002).
- Soil CO_2 efflux can show **distinctive temporal/daily variations** (due to changes in soil temperature or autotrophic contribution). Therefore, the sequence of soil respiration measurements should ideally be fully random in order to avoid any interference with the effect of the climate manipulation being tested. Performing, for instance, all CO_2 measurements at control plots in the morning and all measurements at drought treatment plots in the afternoon could induce serious bias. Also note that calm wind conditions (i.e. low atmospheric turbulence),

which may itself have a specific diel course, may cause biases in flux estimations (Braendholt et al., 2017).

Automated systems (+ high temporal coverage-to-workload ratio, - expensive)

Automated chamber systems can provide year-round high temporal resolution soil CO_2 efflux data. They usually consist of a single CO_2 analyser and one or more auto-chambers, which are operated one after another. Auto-chambers (at least commercial ones) are costly and it is the researcher's task to weigh the pros and cons of the investment. For a mere comparison of annual soil respiration among gradual manipulation treatments (e.g. warming), auto-chamber measurements are mostly not necessary. However, if the climate manipulation has an event character (e.g. heavy rainfall), auto-chambers can provide the needed resolution to capture important flux events. A further advantage is that auto-chamber systems can easily be attached to lasers or field mass spectrometers, thereby allowing high-resolution stable isotope measurements with a series of potential applications (e.g. isotope labelling). Generally, the number of auto-chambers per treatment can be kept as low as possible. Auto-chambers capture the temporal trend in soil CO_2 efflux (spatial variation is captured by periodic manual measurements). Keeping the number of auto-samplers low reduces costs and work-time as these chambers require maintenance and produce large amounts of data to handle. Furthermore, the lower the number of auto-chambers, the higher the measurement frequency of each individual chamber.

Separating autotrophic and heterotrophic soil CO_2 efflux

As mentioned above, separating flux components is often necessary to assess climate manipulation effects on different C pools. There is an array of different methods (Hanson et al., 2000; Kuzyakov et al., 2006; Subke et al., 2006): their applicability depends on the manipulation and the ecosystem. More simple approaches, such as trenching or girdling aim at mechanically excluding autotrophic soil respiration, while more sophisticated approaches such as stable isotope labelling leave plants and soil intact and minimise disturbance. Although the least-destructive method should be the method of choice in climate-manipulation experiments, applicability is often restricted due to financial or methodological constraints. The most common methods are:

- **Trenching** (+ easy to apply; - destructive): trenching incurs the disruption of roots along a trench around a defined plot (from dm^2 to m^2). In the trenched plot, only heterotrophic soil respiration from the decomposition of soil organic matter is anticipated. Autotrophic respiration can be calculated as the difference between control plot respiration and trenched plot respiration. The trench is usually sealed with a plastic foil to prevent root in-growth. Using different sized meshes instead of foil allows the differentiation between root and mycorrhizal respiration (Heinemeyer et al., 2007). Besides the easy and cheap treatment, trenched plots are relatively easy to manipulate. Trenching is therefore frequently used in warming studies. Due to its intrusive nature, the method has several drawbacks such as preventing transpiration from trenched plots, enhanced decomposition of dead roots, and gradually changing soil chemistry due to missing nutrient uptake by roots. All these factors have to be accounted for to produce a

reliable estimate of source contribution (Diaz-Pines et al., 2010; Comstedt et al., 2011) making the method more labour-intensive and sophisticated than at first sight.

- **Tree Girdling** (+ easy; - destructive): as with trenching, girdling is based on the suppression of autotrophic soil respiration. A strip of bark and phloem is removed from the stem to cut-off labile C transport to roots, whereas water uptake is not hampered and roots as well as trees can survive for a while. This method has been shown to provide striking results when applied on a large scale (Högberg et al., 2001), but as most trees die after 1–3 years, the method is less useful in manipulation experiments, which are usually set-up for a longer term. A potential alternative may be stem compression (Henriksson et al., 2015), but this remains to be tested for trees other than pine.
- **Isotope techniques** (+ non-destructive; - expensive and complex): a non-destructive approach in flux partitioning is the use of stable C isotopes (^{13}C) or radiocarbon (^{14}C). Naturally abundant ^{13}C can be used as a measure of autotrophic contribution during distinctive weather events or when C_3 plants grow on C_4 soil or *vice versa* (Ekblad & Högberg, 2001; Kuzyakov, 2006). Alternatively, ^{13}C labelling allows the fate of the carbon taken up during photosynthesis to be traced (Högberg et al., 2008). Labelling studies are the most elegant way of assessing C allocation, but they are more difficult in higher vegetation (trees) that do not easily fit into a chamber. The flux partitioning is also complex. Moreover, the radiocarbon technique is based on different ^{14}C signatures of ambient CO_2 (autotrophic respiration) and respired SOM (heterotrophic). The method is relatively labour-intensive as the ^{14}C signatures of heterotrophic and root respiration need to be assessed several times, and are costly (^{14}C measurements) but can provide good estimates as well (Borken et al., 2006) (not recommended on carbonate soil!).
- **Modelling** heterotrophic efflux (+ non-destructive; - based on simple model): another non-destructive approach is the use of soil C concentrations/stocks and the relationship of heterotrophic respiration to soil temperature and moisture (assessed during lab incubation) as surrogates to model the annual heterotrophic soil CO_2 efflux. Autotrophic efflux is the difference between measured total efflux and modelled heterotrophic efflux. Although this method has proven to produce reliable estimates (Kutsch et al., 2010; Wangdi et al., 2017), it is not recommended for climate manipulation studies where temperature and moisture are the targeted variables during manipulation.

Where to start

Borken et al. (2006), Brændholt et al. (2017), Comstedt et al. (2011), Díaz-Pinés et al. (2010), Ekblad & Högberg (2001), Hanson et al. (2000), Heinemeyer et al. (2007), Henriksson et al. (2015), Högberg et al. (2001, 2008), Hooper et al. (2002), Kutsch et al. (2009, 2010), Kuzyakov (2006), Subke et al. (2006), Wang et al. (2005), Wangdi et al. (2017)

2.2.3.2 Special cases, emerging issues, and challenges

Greenhouse gas efflux through snow

Climate change will affect the magnitude and duration of snow cover and hence wintertime soil microclimate. Cold season soil C dynamics can play an important role and the number of snow-manipulation studies is increasing in temperate to arctic ecosystems (e.g. Wipf & Rixen, 2010; Li et al., 2016).

The preferred approach for measuring greenhouse gas effluc (GHG) fluxes through snow is the concentration gradient method (Sommerfeld et al., 1993). GHG concentrations are measured from the snow to the soil surface. Under uniform snow characteristics, GHG concentrations will linearly decrease from the soil to the snow surface. This concentration gradient, together with specific snow characteristics (porosity and tortuosity) can be used to calculate the GHG efflux. CO_2 concentrations above and in the snow can be easily measured by attaching a straight rod/pipe (inner diameter 2–5 mm) directly to a CO_2 analyser. The rod should have an easy-to-read scale, so that one knows how deep the rod-tip is inserted into the snow. The CO_2 concentration of a specific snow depth can be recorded within a few seconds after inserting the rod into the snow. This way, detailed CO_2 concentration profiles can be measured through deeper snow within a couple of minutes. With shallow snow cover, a single measurement slightly above the snow surface and another measurement at the soil surface can be enough for a flux calculation. For the calculation of the GHG flux, characterisation of the snow profile is necessary. Primarily, snow depth and snow density need to be determined (density by simply weighing a known volume of snow). The gradient method provides accurate flux estimates under less turbulent atmospheric conditions. Under turbulence (wind pumping), a thorough flux estimate can become highly complex (Massmann & Frank, 2006; Seok et al., 2009). The occurrence of ice layers in snow packs can block gas diffusion and complicate flux calculations as well.

Chamber measurements on the snow surface can work well, but can underestimate GHG fluxes, especially when snow is lightly textured (Mast et al., 1998; McDowell et al., 2000; Schindlbacher et al., 2007). Excavating chambers from snow can be an option with shallow snow cover (Björkman et al., 2010). However, excavating chambers into deep snow creates preferred flow paths, which leads to lateral transport of CO_2 and overestimation of the flux. Therefore, excavation, and perhaps even snow disturbance, should be avoided.

It should be noted that most GHG analysers are not built for measurements at temperatures far below freezing: it is therefore recommended that the devices be properly insulated when measuring in the field.

GHG concentrations in the soil profile

Like in snow, soil GHG gradients can be used to calculate the diffusive GHG efflux from the soil surface. The advantage of this approach is that a set of CO_2 sensors, which are installed at different soil depths, can be operated simultaneously, thereby generating soil CO_2 gradients (and fluxes) at extremely high temporal resolution (Maier & Schack-Kirchner, 2014). The method, however, has its drawbacks since the

soil characteristics which are needed for a thorough flux calculation (porosity, tortuosity) are much more difficult to assess and change over time with, for example, soil moisture content. Therefore, the CO_2 gradient method is comparatively rarely applied in soil. GHGs such as N_2O usually do not show a linear concentration profile in soil because they are produced and consumed in soil layers at various depths. Accordingly, a flux calculation is mostly not feasible.

Knowledge of GHG concentrations in the soil profile can, nevertheless, be of advantage in order to improve the mechanistic understanding of climate manipulation effects on soil processes (e.g. how drought affects N_2O production and consumption at different soil depths). Soil GHG concentrations can be easily accessed by inserting capillaries at different soil depths and drawing samples directly with a gas analyser or a syringe for further storage/measurement.

2.2.3.3 References:

Theory, significance, and large datasets

Bond-Lamberty & Thomson (2010, 2014), Bond-Lamberty et al. (2018)

More on methods and protocols

Pihlatie et al. (2013), Pumpanen et al. (2004)

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2.2.4. Soil carbon and nutrient stocks

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Measurement unit: Mg C ha^{-1} or kg C m^{-2}; Measurement scale: plot; Equipment costs: €; Running costs: €; Installation effort: medium; Maintenance effort: -; Knowledge need: medium; Measurement mode: manual

The soil carbon (C) stock is the amount of C stored in the soil (Davidson & Janssens, 2006) and is the product of the long-term balance between C inputs from aboveground and belowground net productivity and C outputs from decomposition and erosion (Kirschbaum, 1995; Melillo et al., 2002). Soil C stocks are sensitive to climate change, because virtually all processes that regulate C inputs and outputs are driven by climate-related factors (such as temperature, CO_2 concentration, rainfall, moisture and nutrient availability) (Tian et al., 2015). Factors other than climate that strongly influence soil C stocks are land-use (Guo & Gifford, 2002) and disturbances, such as erosion (Panagos et al., 2015), volcanic eruptions (Gísladóttir et al., 2010), fire, and human and animal activities (Laurel & Wohl, 2019). The sensitivity of soil C stocks to climatic changes implies that the C sink or source capacity of soils can drastically shift under altering climatic conditions (Kirschbaum, 1995; Melillo et al., 2002; Davidson & Janssens, 2006). Since soils store globally three times more C than the atmosphere (Chapin et al., 2011), these shifts could, in turn, induce powerful feedbacks to the climate system (Kirschbaum, 1995; Melillo et al., 2002; Davidson & Janssens, 2006; Scharlemann et al., 2014; Tian et al., 2015). This is why a detailed quantification of the current global soil C stocks and potential shifts under land-use and climate change are important.

The soil nutrient stock is the amount of nutrients stored in the soil (Chapin et al., 2011), with a focus on nitrogen (N), phosphorus (P), and potassium (K), as these elements are most frequently limiting for plant growth (Sterner & Elser, 2002). Soil nutrient stocks have strong associations with soil C stocks, as nutrients and C are tightly coupled in soil organic matter and are affected by the same processes (Hobbie et al., 2002). Furthermore, plant nutrient availability, which is often linked to soil nutrient stocks (Saynes et al., 2005), has a strong impact on plant growth and litter quality and thus indirectly on the soil C balance (Li et al., 2011).

2.2.4.1 What and how to measure?

Soil C and nutrient stocks are commonly assessed through destructive soil sampling and subsequent determination of the C and nutrient concentration in the soil (excluding particles > 2 mm) (Jobbágy & Jackson, 2000). The sampling is generally performed using manual soil augers. These can differ in type, depending on the soil texture, but have in common that the exact core volume must be known. Augers that strongly disturb the soil volume are thus not suitable for this measurement.

Measurements of soil C are mostly done by dry combustion (Matejovic, 1997; Senesi & Senesi, 2016). Other techniques have also been used in the past (most commonly the Walkley-Black method (wet acidified dichromate oxidation) or loss on ignition), and conversion factors for comparison can be found in Soon & Abboud (1991). A new, less laborious method for laboratory and field determination of soil C, i.e. laser-induced breakdown spectroscopy (LIBS), is currently being developed (Senesi & Senesi, 2016). Methods used for soil nutrient determination depend on the nutrient under investigation. Nitrogen is generally measured together with C using dry combustion (Matejovic, 1997). The other most commonly measured nutrients (P and K) are measured by acid destruction with H_2SO_4, salicylic acid, H_2O_2, and Se (Wallinga et al., 1989). Other important elements (Ca, K, Mg, Na, Mn) can be determined with the same method.

Installation, field operation, maintenance, interpretation

At the sampling spot, the vegetation and the superficial litter layer are removed down to the topsoil layer (Vadeboncoeur et al., 2012). Typically, one core is taken per plot, but in heterogeneous environments it is more desirable to collect a number of smaller cores that are subsequently pooled before analyses and provide a more accurate characterisation of the plot. The latter technique (with more but smaller volumes) yields larger uncertainty in the core volume, and thus in the transformed C and nutrient content per unit area measurements.

To determine the soil carbon stock per unit area, the entire soil depth needs to be sampled. A core is taken down to the bedrock and is split up into different depth layers to obtain information about the depth profile of the stocks (Maillard et al., 2017). In general, a subdivision is made between topsoil (0–10 cm depth) and subsoil (> 10 cm depth), but in some cases, it might be important to use a finer scale. Alternatively, soil horizons are used as subdivisions instead of fixed soil depth (Maaroufi et al., 2015). For more information on the importance of sampling design, the spatial scale of the sampling, and sampling depth, one can consult Maillard et al. (2017) and Allen et al. (2010).

Soil samples are sieved at 2 mm to exclude stones and roots and dry weight is determined (optimally the samples are dried at a maximum of 40 °C to avoid volatilisation of N). Subsequently, the C and nutrient content of finely ground aliquots is determined (Matejovic, 1997). Finally, the C and nutrient stocks per unit area can then be calculated by applying the following equation:

$$S = \frac{\sum_{i=1}^{n} C_i * DW_i}{A}$$

where S is the C or nutrient stock over the entire soil depth, which is split into n layers. C_i is the C or nutrient concentration for layer i (expressed in%), DW_i is the dry weight of the core taken from layer i, and A is the surface area of the core.

Alternatively, the C and nutrient stock can be expressed per volume instead of per area. This is necessary when it is not desirable or possible to sample the entire soil depth. In that case, the C or nutrient stock of the soil layer of interest (S_i) is calculated using the volume of the sampled core (V_i):

$$S_i = \frac{C_i * DW_i}{V_i}$$

Where to start

Davidson & Janssens (2006), Jobbágy & Jackson (2000), Maillard et al. (2017), Melillo et al. (2002), Senesi & Senesi (2016)

2.2.4.2 Special cases, emerging issues, and challenges

Stony soils

When calculating the C and nutrient content in stony soils, the stone volume (stones > 2 mm; Novák et al., 2011) of the soil should be taken into account, as the volume occupied by stone is assumed not to contain a significant amount of C (Rytter, 2012). The correction is generally made with the 'quantitative pit method' (Vadeboncoeur et al., 2012), where the rock fragment density is estimated by digging a pit with dimensions large enough to obtain a representative sample of the soil (< 2 mm) and stone (> 2 mm) volume. Subsequently, the volume of the pit and stones are measured. For the pit volume, the pit is lined with plastic and the volume of water necessary to fill it up to the soil surface is tracked (Vadeboncoeur et al., 2012; Mehler et al., 2014; Beem-Miller et al., 2016). The volume of stones is estimated by the water displacement method or by using a hydrostatic scale (Mehler et al., 2014).

In cases when the quantitative pit method is unsuitable (e.g. due to its destructive, labour-intensive or costly nature), other, but less accurate methods can be used. The most common alternatives are the hammer, hydraulic push, and rotary coring methods (Beem-Miller et al., 2016).

Coarse roots

In soils containing a substantial amount of large roots, the same technique can be used as in stony soils (see above) to exclude the root volume from the soil volume (Vadeboncoeur et al., 2012; Mehler et al., 2014). In soils with small roots (e.g. grasslands), the root volume is generally neglected.

Carbonate-containing soil

Soils on calcareous bedrock contain inorganic C in the form of carbonates (e.g. calcite or dolomite). Methods assessing soil C, such as dry combustion or wet oxidation, provide a bulk measure of total soil C consisting of organic and inorganic C components. In most climate-manipulation experiments the primary interest is on how organic C is affected. Therefore, in order to assess effects on organic C, inorganic soil C content needs to be subtracted from total soil C. Under specific conditions, climate manipulations could not only affect organic C, but also directly affect the dissolution or formation of soil carbonates (e.g. their pedogenic formation due to precipitation manipulation in arid or semi-arid environments).

Carbonate contents (and consequently their share in organic C) can be assessed in several ways. Most approaches are based on the comparison of a soil sample containing organic + inorganic C (= total soil C) with a sample free of inorganic C. The technically easiest way of assessing the carbonate content is to treat soil samples with HCl and then conduct a volumetric determination of the released CO_2 (Nelson & Sommers, 1982). This gasometric approach is called the 'Scheibler' or calcimeter method. The calcimeter method is straightforward, albeit time and labour-consuming, because only a limited number of samples can be treated simultaneously. To assess the carbonate content of a higher number of samples simultaneously, acid washing (e.g. Midwood & Boutton, 1998; Schnecker et al., 2016) or acid fumigation (Harris et al., 2001; Walthert et al., 2010) can be applied to remove carbonates from the samples. These techniques have the advantage that the soil samples can also be used for further analyses (e.g. for isotope measurements). While acid washing can lead to loss of acid-soluble organic C, acid fumigation has been successfully used with calcite (Harris et al., 2001) and even dolomite (Walthert et al., 2010) without solute C loss. Beside these methods, carbonate content can also be assessed non-destructively by FT-IR spectroscopy (Tatzber et al., 2007) or near-infrared analysis (NIRA) (Ben-Dor & Banin, 1990). Such methods are, however, comparatively sophisticated and may be applied only when a detailed characterisation of organic C is foreseen anyway. The thermal gradient (ThG) method has been shown to perform well, especially with regard to the assessment of dolomite contents (Vuong et al., 2016).

2.2.4.3 References

Theory, significance, and large datasets

Davidson & Janssens (2006), Kirschbaum (1995), Melillo et al. (2002), Scharlemann et al. (2014)**,** Tian et al. (2015)

More on methods and existing protocols

Allen et al. (2010), Jobbágy & Jackson, (2000), Maillard et al. (2017), Senesi & Senesi (2016); Vadeboncoeur et al. (2012)

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2.2.5. Nutrient mineralisation

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Measurement unit: µg N or P g^{-1} soil (or g m^{-2}; ug N g^{-1} SOM); Measurement scale: plot; Equipment costs: None - €; Running cost: € - €€; Installation effort: low; Maintenance effort: medium; Knowledge need: medium to high; Measurement mode: manual

Nutrients are essential for plant growth. Mineralisation is the conversion of dead organic matter (OM) to inorganic forms of, for example, N, P, K, and Ca by soil microbes. Consequently, mineralisation is the process responsible for making organic compounds re-available for plant uptake, and thus the rate of mineralisation is a critical component of ecosystem productivity. A measurement of soil nutrient pools merely supplies a snapshot of the available nutrient pool at a given time; it does not provide information on nutrient flow-rates from OM to microorganisms and plants.

It is difficult to quantify gross mineralisation rates directly in soils, as soil microbes and plants may take up a substantial part of the mineralised nutrients immediately after nutrients become available within the soil. Consequently, we focus on net mineralisation methodologies in this chapter.

Net mineralisation is the balance between a) gross mineralisation of organic matter to inorganic mineral forms and b) immobilisation of mineral nutrient forms. Immobilisation is the inverse process of mineralisation, where soil microbes take up inorganic nutrients and incorporate them into microbial biomass, making those nutrients unavailable to plants. However, immobilisation can also be physical, for example when nutrients form in mineral complexes within the soil.

Climate change directly affects microbial activity through changes in soil temperature and moisture regimes and thus induces changes in microbial decomposition activities and availability of soil nutrients for plants. Therefore, measurements of net mineralisation rates are critical for linking soil–plant interactions in climate- or global-change manipulation experiments as well as in non-manipulated natural experiments across space (i.e. along gradients) and time (i.e. observational studies).

2.2.5.1 What and how to measure?

Basically, net nutrient mineralisation is the difference in nutrients in the soil or on an ion exchange membrane at two time points. There are different methods available for estimating mineralisation but none of them are optimal (see Table 2.5.1.1). Most commonly used include the buried bag technique, ion resin exchange, and pool dilution.

Table 2.5.1.1. Comparison of three methods for estimating nutrient mineralisation.

	Methodology	**Pros/cons**	**Intrusiveness**
Buried bags	Two soil cores are sampled. One goes to the laboratory for analysis of initial nutrient content. The other is incubated in the soil in a plastic bag and nutrients in the core are measured after incubation. Mineralisation is the difference in nutrient content before and after incubation.	Cheap and easy to use. The method only takes fluctuation in soil temperature into account but soil moisture is kept constant during incubation. Microbial immobilisation of nutrients in the bag may mask the results. Mineralisation is uncoupled from plant processes.	Two soil cores per mineralisation period.
Ion exchange membranes	Membranes for absorption of anions and cations, respectively, are inserted in slits in the soil made by a knife.	All the major nutrient elements can be extracted simultaneously with one extraction. Includes spatiotemporal variation in temperature and soil water. Removal of nutrients from the resins has been reported from high pH soils.	Less destructive. The same slit can be used for sequential incubation and harvesting of membranes.
^{15}N pool dilution	^{15}N pulse is added to a soil as $^{15}NH_4$, $^{15}NO_3$, or other N forms. The excess ^{15}N to the natural abundance is followed in different pools over time by soil sampling. The dilution of ^{15}N mirrors the mineralisation and release of nitrogen.	The pool dilution method gives a more mechanistic understanding of the underlying processes as the fate of a cohort of ^{15}N can be followed over time. Time-consuming and more expensive analysis.	At least 3 soil cores at three different times: T_0, T_1, and T_2.

Buried bag method

Net mineralisation rates of, for example, N and P can be measured in the field by the buried bag method (Eno, 1960). Net mineralisation measured in buried bags is an estimate of the balance between mineralisation and microbial immobilisation of nutrients in the absence of plant roots as the technique prevents plant uptake of mineralised nutrients, but allows uptake by microorganisms. The method accounts for the differences and fluctuations of *in situ* soil temperatures during the incubation period, while the water content is kept constant inside the bag. Thus, one should note that the net mineralisation rates obtained via this method incur destructive soil sampling and do not account for plant interactions, leaching processes, and lateral flow.

Duplicate soil cores are sampled per plot or across a site per mineralisation period. One intact core is carefully placed in a sealed low-density polyethylene bag (commonly available as lunch bags in supermarkets), making sure that the core does not break, and the bag is then placed back into the ground for a period of weeks to months, depending on the season and research objectives. The remaining soil core is transferred to the laboratory where inorganic N (NH_4^+ and NO_3^-) and P (PO_4^-) are measured following extraction (see section 2.2.1.1 in protocol 2.2.1 Soil microbial biomass - C, N, and P for handling of soil samples and extraction). After the desired incubation period has passed, the bag is retrieved from the ground and inorganic nutrients are extracted in the same way as the initial soil core. The net mineralisation rate is the difference in inorganic N (NH_4-N, NO_3-N) in the non-incubated soil core relative to the bag-incubated core, and it is expressed per unit time (e.g. µg NH_4-N $month^{-1}$). Seasonal and annual mineralisation rates can be estimated from sequential incubations running over a growing season or a full year.

In nutrient-poor soils, the microorganisms may immobilise a substantial amount of the mineralised nutrients in the bags (Schmidt et al., 2002). Therefore, including measurements of microbial immobilisation, i.e. microbial biomass changes, within the incubated bags enables a better estimate of total mineralisation. However, it is also a substantial amount of extra work. This can be done by measurement of microbial biomass C, N, and P before and after incubation. For more details see Schmidt et al. (1999, 2002) and protocol 2.1.1 Aboveground plant biomass and 2.2.1 Soil microbial biomass – C, N and P.

Ion exchange resin membranes (IEMs)

Plant-available soil nutrients can be estimated using ion exchange membranes (IEMs). Previously, ion exchange resin beads were used to adsorb soil solution cations and anions, simulating plant-root surface properties with adsorption of nutrients directly from the soil (Giblin et al., 1994). However, using resin beads is destructive, similar to the buried bag method, and resin bead measurements do not always correlate well with other mineralisation measurements (Giblin et al., 1994). Recently, ion-exchange membranes (IEMs) have emerged as the preferred method for estimating mineralisation: its 2-dimensional structure imposes minimal disturbance to plots and soil, while allowing for full membrane contact with the surrounding soil medium (Harrison & Maynard, 2014). Consequently, strong relationships have been reported for soil N availability obtained using IEMs and classic soil extraction and mineralisation methods (Duran et al., 2013). Furthermore, IEMs are relatively easy to prepare in large numbers, can be cut to any size, and they are cost-effective to produce in bulk, thus making it possible to cover a large area *in situ* and perform long-term continuous mineralisation measurements by repeated insertion within the same micro-site (Harrison & Maynard, 2014).

IEMs work much like their resin bead counterparts (Qian & Schoenau, 2002). Large anion and cation membrane sheets are commercially available as they are commonly used in water treatment facilities. Once cut to the desired size, anion and cation strips are kept separate and charged in an appropriate solution prior to insertion. Typically, cation strips are charged with 0.5 M HCl and anion strips are charged with 0.5 M $NaHCO_3$ (Lajtha et al., 1999). After charging, the IEM strips should be rinsed with H_2O to remove any excess chemical from the IEM surface. To maximise soil contact, the IEM strips are inserted into the ground at a slight 15–30 ° angle (Lajtha et al., 1999). Note that the ion-exchange method is sensitive to moving soil water, as nutrients are adsorbed from passing soil water (Binkley, 1984).

Depending on the research objective and ecosystem type, the IEMs can be inserted for days, weeks, or months before retrieval, but note that Lajtha et al. (1999) recommends relatively short incubation times in order to minimise potential desorption occurring if the IEM nutrient concentration becomes greater than the soil surroundings. This can take weeks during summer but several months during winter. This reverse gradient phenomenon has previously been observed for ion-exchange resin beads, suggesting either microbial removal of adsorbed nutrients or a reverse gradient caused by prolonged IEM immobilisation (Giblin et al., 1994). If longer-term mineralisation rates are desired, sequential insertion of IEM strips into the same slit in the ground is possible.

Once retrieved from the field, IEM strips should be kept cool or frozen before desorbing anions and cations by extraction in the lab.

Plant root simulator (PRS) probes are an alternative commercial option to custom-made IEM strips. PRS probes have similar properties to custom IEMs, and they are usually bought with a prepaid nutrient extraction from the manufacturer, omitting the labwork component for the user. Similar to custom IEMs, PRS probes consist of paired negatively and positively charged IEM strips that are inserted into the soil. Once retrieved, the probes are sent to a laboratory where a large range of nutrients can be measured.

Isotopic tracer – ^{15}N fate or pool dilution method

It is possible to determine gross N transformation rates by applying a trace amount of inorganic ^{15}N to a soil using the pool dilution method (Davidson et al., 1991; Murphy et al., 2003; Poertl et al., 2007). Based on a time series of the isotopic signature of the different soil N pools over time, gross mineralisation can be calculated using either an equation related to the decline of the tracer (Kirkham & Bartholomew, 1954, 1955) or with a simulation model (Wessel & Tietema, 1992; Mary et al., 1998).

The knowledge on gross transformation rates using the pool dilution technique gives a better mechanistic understanding of the underlying processes (Verchot et al., 2001). However, the method is very time-consuming and application throughout a year for annual estimates of nutrient mineralisation has never, to our knowledge, been applied.

Where to start

For details on the classic buried bag method, see Eno (1960). For a comprehensive review on ion-exchange techniques, see Qian & Schoenau (2002), and for modern IEM-specific methodologies, see

Duran et al. (2013) or Lajtha et al. (1999). Murphy et al. (2003) provide details on the ^{15}N pool dilution method and a good overview on the N transformation processes and key references.

2.2.5.2 Special cases, emerging issues, and challenges

Comparison of buried bag and IEM methods

The different approaches for estimating net mineralisation rates in soil have their own pros and cons – none of the methods are generally considered optimal (Table 2.5.1.1; Binkley, 1984; Binkley et al., 1986). IEM strips are inserted into the ground so that they are in direct contact with the surrounding soil environment. This is in contrast to the buried bag method, where the incubated soil is isolated from any fluctuations in water content, precipitation, or movement of soil water. Additionally, the buried bag method disregards root activity, whereas IEMs are inserted into the soil matrix where nutrient sorption "competes" with roots and microbes alike. In contrast to IEMs, the buried bag method can be used to estimate and account for microbial immobilisation of nutrients. Furthermore, the buried bag method is not subject to the reverse-gradient bias, described above, which may occur during prolonged IEM incubation times. Compared to the buried bag and ion-exchange resin bead methods, IEM strips are minimally destructive, and can be inserted repeatedly at the microsite level. The buried bag method requires duplicate cores to be compared, which in heterogeneous soil environments can add potential noise to the data. In contrast, when dealing with small-scale heterogeneity, multiple small IEM strips can be relatively easily inserted across plots or sites to account for spatial variability.

Amino acids

The methods described here were originally developed for measuring inorganic nutrients. However, experiments have demonstrated that many plants are able to take up amino acids (Kielland, 1994; Näsholm et al., 1998; Nordin et al., 2004; Schimel & Bennett, 2004), and ericaceous species can even use more complex N-containing compounds due to their mycorrhizal association (Read & Perez-Moreno, 2003). Therefore, quantifying amino acid availability or flux-rates may be desired. The buried bag method allows for extraction and quantification of dissolved organic N (DON), which is total dissolved N minus inorganic N forms. DON includes amino acid components and DON can therefore be used as proxy for amino acid availability. Similarly, extractions of IEM strips can also be analysed for DON.

2.5.5 References

Theory, significance, and large datasets

For details on the classic buried bag method, see Eno (1960). For a comprehensive review on ion-exchange methods, see Qian & Schoenau (2002), and for modern IEM-specific methods, see Lajtha et al. (1999) and Duran et al. (2013). Murphy et al. (2003) provide details of the ^{15}N pool dilution method and a good overview of the N transformation processes and key references.

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2.2.6 Foliar litter decomposition

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Measurement unit: mass loss %; Measurement scale: plot or site; Equipment costs: €; Running costs: €; Installation effort: low; Maintenance effort: low; Knowledge need: low; Measurement mode: manual

Decomposition is the breakdown (biological and chemical processes) and leaching of soluble compounds (physical process) of plant litter such as foliar, woody, and root litter, as well as of soil organic matter (SOM), with subsequent mineralisation and humification of organic compounds. It is a major determinant of carbon and nutrient cycling in ecosystems and a key driver of soil formation and fluxes of elements, such as carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) into the atmosphere. An estimated 60 Pg CO_2 is emitted annually by the decomposition of litter and soil organic matter (Houghton, 2007). Hence, litter turnover is of significant importance for the global carbon dioxide balance and is a proxy for ecosystem function. Anticipated increases in atmospheric CO_2 as well as nitrogen (N) deposition might affect the quality of foliar litter (Norby et al., 2001) and climate change may substantially affect litter decomposition rates. However, considerable uncertainty remains in the decomposition patterns of plant litter and thus in the estimates of carbon dioxide emissions from the litter pool as well as in the size of the residual soil organic matter (SOM) relevant for carbon (C) dynamics of ecosystems.

2.2.6.1 What and how to measure?

The most commonly used approach to determine litter decomposition in all ecosystems is the litterbag method (Bocock & Gilbert, 1957). This method allows for measuring litter mass loss over a certain period and thus estimates decomposition rates. Litterbags can be prepared using site-specific litter (using one plant species or representative plant community mixes of litter from different species) or standard substrate.

Litterbags with site-specific (local) litter

Studies that use site-specific litter are commonly used to determine local litter decomposition since the decomposer communities are often adapted to the site-specific litter. The approach requires the collection of newly shed and intact mature shed leaves, the drying and preparation of the sampled litter, preparation of litterbags, weighing of the samples, installation of the litterbags in the field, and retrieval of incubated litterbags at given times. In forest ecosystems, recently senesced litter (leaves/needles) is collected during autumn, or during the dry season in tropical ecosystems with litter trap nets (e.g. 0.25 m^2 and 1 mm mesh size; for more information see the ICP Forests Manual-Part XIII, 2016). An alternative is to use spread-out tarpaulins, which collect foliar litter across a larger surface or to gently shake shrub branches until leaves are shed directly into a bag. In grassland ecosystems, standing litter should be collected before winter by direct cutting from the plants. Loose plant material should be avoided, because it can be unclear if the litter is from this or a previous year. The newly collected foliage litter should be dried prior to incubation at 50 °C until constant weight. After incubation, the litter should be dried at the same temperature for 24 to 48 h in a ventilated oven.

Litterbags can be constructed from netting with different mesh sizes allowing size-selective exclusion of consumers: mesh size < 1 mm includes only microorganisms; mesh size > 1–2 mm includes meso- and micro-fauna; mesh size > 2 mm includes macro-, meso-, and micro-fauna; Bradford et al., 2002; Karberg et al., 2008). Polyester or high-density polyethylene nets for light-intensive sites are used for making bags. The litterbag size should be appropriate to the litter-size and is commonly 10 x 10 or 20 x 20 cm. Litterbags generally contain between 1–3 g of intact (not ground) material. The weight of the initial litter content with the litterbag should be recorded as precisely as possible (preferably to the 3rd or 4th decimal place). During transport to and from the field, it is important that the bags are not damaged and that no litter is lost. This may be taken care of by transporting the litterbag in paper envelopes. Label each bag with a unique identifier (e.g. metal plate with ascending numbers), or with a plastic tag giving the weighed litter mass. Metal plates will allow you to detect the bags with a metal detector at the end of the field-incubation period.

Litterbags with standard material

Common litterbag studies are most often conducted based on site-specific litter, making comparisons of different experiments and sites challenging. This is also partly due to the lack of common protocols and standard matrices. In the past, there have been several attempts to use a standardised material (e.g. cotton-strips (Latter & Howson, 1977), cellulose sheets (Rosswall & Veum, 1971)) for the estimation of litter decomposition. However, such materials are not representative of foliage litter decomposition.

Recently, the use of commercially available teabags as a pre-made “litterbag” with standardised litter material was introduced by Keuskamp et al. (2013). The Tea Bag method is a well-standardised, cost and time efficient method that provides proxy data on decomposition rates and litter stabilisation. Two types of tea material are used: i) green tea which is relatively labile and thus characterised by a higher decomposition rate and ii) rooibos tea which contains more structurally complex compounds and is characterised by a relatively low decomposition rate. It is crucial that the same tea content is used, therefore always check the EAN number when purchasing tea (green tea – Lipton Indonesian tea Sencha tradition: EAN 87 22700 05552 5 and rooibos tea: Lipton herbal infusion rooibos: EAN 87 22700 18843 8.

This common metric provides a reliable tool for inter-site and even global comparisons both in experimental and observation contexts (Borer et al.,2014; Verheyen et al., 2016). Decomposition of teabag material should be viewed as a proxy for decay rates. As such, the teabag method does not give a quantitative measure of local C losses and decomposition rates, since the tea is not equivalent to the site-specific litter, but it can be related to local rates (e.g. by simultaneous incubation of site-specific litter) and modelling.

Protocols for weighing samples and selecting plot sizes are the same as for standard litter.

Where to start

Berg (2000, 2014), Keuskamp et al. (2013)

Installation and field operation and interpretation

The start of incubation should be co-ordinated across the sites. The incubation length, however, depends on the research question. While the early decomposition stages (ca. 0–40% mass loss) are characterised by the mineralisation of preferential labile C sources and by leaching, the later stages (ca. 40–100% mass loss) are determined by mineralisation of recalcitrant C. Moreover, driving factors of different decomposition stages vary or change their directional effect (Berg, 2014). Since most climate simulation models need the temporal dynamics of litter C and nutrients, it is desirable to focus on medium- to long-term litter decomposition until the limit values (stage where litter mass is either stabilised or decomposes extremely slowly; Berg, 2000) are captured (i.e. incubation length > 1 year or more) and to include several sequential samplings.

Select homogenous replicate areas with uniform vegetation type, topography, and soil type, since any deviation in those parameters will influence the decomposition rates. Generally, we recommend that there should be at least three replicate areas per site or treatment with at least five replicate litterbags per replicate area and sampling point. The optimal plot size depends on the experimental set-up and ecosystem type. Usually, the plot size must be larger for forest ecosystems (e.g. 25 x 25 m) than for grassland ecosystems (e.g. 3 x 3 m). However, in experimental plots the size will be determined by the experimental set-up.

Litterbags can be incubated on the surface or buried into the upper soil layer (0–5 cm; Knacker et al., 2003). The choice of exposure can influence the litter decomposition, and for long-term decomposition trials with between-site comparisons, burial into the soil is preferred. The incubation layer should be marked. Samples should preferably be installed in straight lines in order to capture the same topography. Further, we advise installing the bags in the order of the planned sequential retrieval, to allow easy identification in case the labels get lost. Once the bags have been installed, the bags should be marked with a stick (place all sticks on the same side of the respective bags) and the lay-out should be sketched to aid the detection of the bags at the time of retrieval. Also record the date of the installation.

The retrieval date should be planned in relation to the date of installation. Make notes if the bags are damaged or already dug out as this is of relevance for data processing. Each litterbag should be placed in a separate plastic bag and relabelled if the initial labelling is unreadable. The litterbags should be processed as soon as possible. If they cannot be processed immediately, then the samples should be stored at -18 °C to prevent further decomposition. Remove all soil and root debris from the surface of the bags. Dry the bags at 50 °C until constant weight. Note the weight of remaining litter (without bag). Store the collected litter in paper bags (label them) for further analyses.

If the collected litter material is visibly contaminated with soil or the remaining weight is higher than the initial weight, then the ash content (i.e. the mineral residue after removal of organic matter by ignition) of the initial litter as well as after incubation should be determined by heating in a muffle oven at 500 °C for 16 h, to correct for the mineral part (Harmon et al., 1999; Soil Survey Staff, 2004). The ash weight can then be subtracted from the total dry weight in order to obtain the real end mass of the litter after incubation. Alternatevily, remainig carbon should be alanlysed. If roots and/or decomposers have entered the bag, all visible roots and/or decomposers should be removed manually.

Litter mass loss is calculated as the difference between the initial dry mass and the final dry mass after the field incubation.

$$\text{Mass loss } (\%) = \frac{((m_0 - bag) - m_1)}{(m_0 - bag)} \times 100$$

where:

m_0 = weight of the initial litter with bag (g)

bag = weight of the empty litterbag (g)

m_1 = weight of the litter at a given sampling point (g)

To interpret the mass loss, further parameters are needed. The minimum requirement would be to link the obtained mass loss to site climatic parameters (mean annual temperature, mean annual precipitation, annual mean temperature amplitude) and basic soil parameters (pH in $CaCl_2$, organic carbon, total N, P, K, Ca, Mg, Mn). To understand the nutrient release processes during decomposition of soil and litter, nutrient data in the litter before and after incubation (N, P, S, K, Ca, Mg, Mn, lignin) will be needed. For this, a multi-element extraction should be applied (Pleysier & Juo, 1980). In optimal cases, it would be advisable to take continuous measurements of litter temperature and moisture using sensors within litterbags to record local climatic conditions (Wang et al., 2015). When litterbags are made with isotopic labelled litter, measurement of ^{13}C and ^{15}N through the decomposition process will provide valuable insights into the involvement of microorganisms in the decomposition process (e.g. Djukic et al., 2013). Also, combining litterbags with experimental studies (e.g. rain maniputlation, soil fertilisation) or elevation gradients (e.g. tree-line shifts) will allow a better understanding of other global-change drivers relevant for decomposition processes. Similarly, involving litterbag studies in monitoring studies such as soil carbon stocks inventories (e.g. FAO Soil Carbon Partnership, national soil inventories) or belowground diversity observations (e.g. GEO BON) will provide a powerful link between the potential drivers and functions, which is of particular interest for interpreting the effects of climate and biodiversity change.

A wide range of carbon and ecosystem models already use a decay process (typically with different representations of the decay process based on molecular structure and chemical composition of the litter that decomposes at unique rates, etc.) to simulate C dynamics. To run a simple dynamic C model (e.g. Soil Carbon Model – Yasso, 2017) information on chemical AWEN fractions – i) compounds hydrolysable in acid, ii) compounds soluble in water, iii) compounds soluble in a non-polar solvent (ethanol or dichloromethane), and iv) compounds neither soluble nor hydrolysable – of the litter type should be determined if they are not listed on the Yasso website (Yasso, 2017).

Where to start

Harmon et al. (1999), Knacker et al. (2003), Pleysier & Juo (1980), Soil Survey Staff (2004), Wang et al. (2015), Yasso (2017)

2.2.6.2 Special cases, emerging issues, and challenges

In ecosystems where litter decomposition is strongly affected by the activity of macro- and meso-fauna (e.g. tropical ecosystems), incubation of litterbags with different mesh sizes is recommended in order to obtain the best information on litter decomposition dynamics (Powers et al., 2009).

Annual water-level fluctuations affect litter decomposition in wetlands: therefore frequent sampling that covers all variations during litter decomposition over a long-term is essential for the fundamental understanding of decomposition processes in these environments.

In semi-arid ecosystems, photodegradation is one of the controlling factors of aboveground litter decomposition. If litterbags are incubated on the surface, high-density polyethylene nets should be used for the litterbag in order to inhibit bag degradation. Moreover, regardless of the material type used to construct the litterbags, the % of UV transmittance should be measured and reported. Soil erosion by water or wind can also affect litter decomposition in arid and semi-arid ecosystems, as well as interact with photodegradation (Throop & Archer, 2008). So, these novel drivers (i.e. photodegradation, non-rainfall water sources, fragmentation by soil transport) of litter decomposition in arid and semi-arid ecosystems have to be considered when designing experiments in these regions.

When using teabags to investigate litter decomposition, one should bear in mind that the tea material (especially green tea) is prone to leaching, which in turn has to be considered when incubating bags and analysing data (Djukic et al., 2018).

2.2.6.3 References

Theory, significance, and large datasets

Berg & McClaugherty (2003), Powers et al. (2009), Prescott (2010), Tuomi et al. (2009), Vitousek et al. (1994)

More on methods and existing protocols

There have been many litterbag studies, providing a good source for different methods and protocols:

- Long-Term Intersite Decomposition Experiment (LIDET): https://andrewsforest.oregonstate.edu/sites/default/files/lter/pubs/webdocs/reports/lidet.htm
- Global Litter Invertebrate Decomposition Experiment (GLIDE): http://wp.natsci.colostate.edu/walllab/research/global-research/global-litter-invertebrate-decomposition-experiment/
- The Detrital Input and Removal Experiment (DIRT): https://dirtnet.wordpress.com
- TeaComposition - A Global Litter Decomposition Study: http://teacomposition.org

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2.2.7 Root decomposition

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Measurement unit: mass loss %, nutrient loss %; Measurement scale: root segments of individual plants; Equipment costs: €; Running costs: €; Installation effort: medium to high; Maintenance effort: low; Knowledge need: low; Measurement mode: manual (and data loggers)

In many ecosystems, roots are one of the dominant carbon and nutrient inputs into the soil. It is estimated that roots can comprise between one and three quarters of total plant input to the soil (Robinson, 2007; Freschet et al., 2013). Roots also directly interact with the soil and can start to decompose within the protection of soil aggregates and fine pores, serving to stablise soil organic matter and sequester C (Jones & Donnelly, 2004; Schmidt et al., 2011): an important contribution to offsetting greenhouse gas emissions (Marian et al., 2017). Generally, roots are more recalcitrant than leaf litter due to higher concentrations of lignin and suberin and decompose on average 1.5 to 2.8 times slower (Silver & Miya, 2001; Rasse et al., 2005; Freschet et al., 2013). Rates of root decomposition parallel those of leaf litter decomposition, and are affected by, amongst others, climate, litter quality, edaphic conditions, and the soil microbial community (See et al., 2019). Nevertheless, due to possible differences between aboveground plant parts and roots, it is necessary to study roots as a separate component to gain a more complete understanding of processes influencing plant litter decomposition. Moreover, unlike leaves, roots are buffered from changes in surface temperatures and other disturbances (e.g. erosion, herbivory, fire). Decompsosing roots belowground may therefore respond differently from surface leaf litter to global drivers such as climate and land-use change.

Studying root decomposition presents several challenges. Most notably, any extraction of roots from the soil to determine decomposition radically alters the rhizosphere interaction that could have otherwise influenced the decomposition process. This differs from studying leaf litter decomposition where material can be collected and returned to the soil surface with minimal disturbance. The challenge of disturbing roots to understand decomposition and other belowground processes has driven the development of techniques to study roots *in situ*. These techniques include rhizotrons, isotope labelling, x-ray tomography, and DNA-based probing (see Bardgett et al., 2014). However, many of these techniques are expensive, so the traditional approach of collecting root litter remains the predominant form through which root litter decomposition is studied. Root litter decomposition studies have been used in several climate-change experiments from *in situ* warming experiments (Liu et al., 2017) to space-for-time subsitutions across climatic latitudinal and elevational gradients (Marian et al., 2017). Nevertheless, root litter approaches outlined in this chapter can equally be used to investigate other major drivers of root decomposition such as land-use change (Smith et al., 2014).

2.2.7.1 What and how to measure?

Similar to aboveground litter outlined in the protocol 2.2.6.1 in the Foliar litter decomposition, the litterbag approach is quick and cheap and facilitates either a large spatial or temporal (e.g. many harvests) scale study. However, root decomposition studies usually use site-specific or pot/common

garden grown litter rather than standardised litter types that can be used in leaf litter studies. This is because root-litter quality can vary by several orders of magnitude more than leaf litter (Freschet et al., 2017). Growing in the soil, roots have high plasticity at a micro spatial and temporal scale that can lead to large variations in physiochemistry and morphology across the root network of an individual plant and across species within a plant community. For example, many species have roots colonised by mycorrhiza and the extent of this colonisation has been shown to accelerate root litter decomposition (Hodge et al., 2001; Langley et al., 2006) or to slow down the decomposition of colonised root tips (Goebel et al., 2011). Therefore, any root decomposition must provide as much information as possible regarding the source and type of root litter used.

Root-litterbag approach

Roots for litterbags can either be collected from (semi-)natural communities, common gardens, or pots. Ideally, the environmental conditions for rearing roots in pots or common gardens should aim to emulate as far as possible the conditions experienced by the natural community (e.g. soil type and texture, water regime, temperature, mycorrhiza, soil herbivory). Nevertheless, reared roots are typically "spoilt". Roots in pots are grown in soil with low compaction, minimal herbivory, and typically have younger root systems, higher specific root areas, and are enriched in nutrients compared to roots from natural communities (Freschet et al., 2017). Results from decomposition studies using pot-grown roots therefore should be interpreted with caution.

When harvesting root material from natural and (semi-)natural communities, it is necessary to document plant species, timing of the year (e.g. period of peak root death in winter or dry seasons), soil depth, and management practices. Root material should be collected from multiple stands of vegetation to capture variation in root litter quality. Unless working with monocultures or underneath a single tree species without ground flora, it is necessary to extract a soil turf where the roots can be traced back to the intended plant species. In grassland and shrubland ecosystems, root production is usually concentrated in the upper 10–50 cm of the soil profile, therefore a soil turf of the correct depth should be extracted from which to harvest root material. Given limited evidence of nutrient resorption following root senescence, it is generally viewed as acceptable to use a mixture of living and dead roots to investigate root decomposition (Aerts, 1990; Silver & Miya, 2001; Freschet et al., 2013).

After harvesting a plant–soil turf or soil, roots can be washed free of any adhering soil. This is best achieved using a series of fine sieves with the finest sieve around 500 μm (0.5 mm) to collect the majority of visible roots (Livesley et al., 1999).

The collected root material can be sorted into desired functionality for the aims of the decomposition study. The majority of studies primarily focus on decomposition of fine-roots as these are believed to exhibit the fastest C turnover as well as being responsible for plant nutrient acquisition. Fine-roots are defined either by following an arbitrary diameter threshold (most commonly fine roots are defined as < 2 mm in diameter), or according to their branching position (i.e. the first-three root orders), following their function, absorptive, and transport roots (Goebel et al., 2011; McCormack et al., 2015; Freschet & Roumet, 2017). Selection of fine-roots will exclude rhizomes and taproots. Equally, selection of roots of the first-three orders will exclude the main anchoring root system. It is worth noting that when studying herbaceous roots with < 2 mm diameter cut-off, the majority of the root system is likely to be < 1mm in diameter. Therefore, providing

information on root litter diameter is useful for interpretation of the results, especially with studies based on functional criteria still in their infancy.

Once sorted, root material can be pooled and oven-dried between 60 and 70 °C for 48 h to a constant weight. After drying, material can be coarsely chopped (i.e. into 2 to 3 cm sections) for thorough homogenisation. Homogeneity is important when collecting roots from a whole plant and/or from multiple stands across a community or landscape. Unless specified as part of the study aims, this pooling is important to ensure there are no biases in root properties, for example, in relation to collection depth or edaphic conditions.

Root decomposition studies typically use finer mesh sizes than leaf litter studies to exclude the ingrowth of new roots into litterbags. The smallest root diameter is around 50 µm (0.05 mm); however, mesh sizes used in root decomposition studies can range from 0.05 mm to 5 mm (Silver & Miya, 2001; Freschet et al., 2013). To exclude the ingrowth of new roots it unfortunately often requires the exclusion of most soil micro- and meso-fauna. Given our experience for short term studies (i.e. months to a year) fresh root ingrowth may be minimal, sporadic or visible (i.e. white roots) allowing the use of larger mesh sizes that permit soil fauna access (Smith et al. 2019). An alternative approach, typically used in tropical grasslands, is to place root litter into buried pots that are covered with mesh and inverted to prevent root ingrowth from plants on the soil surface but can facilitate entry of micro- and meso-fauna. However, Silver & Miya (2001) found that the buried pot approach yielded significantly lower root decay rates, presumably from the greater exclusion of living roots and rhizo-depositon that stimulates the microbial decomposition process. Other approaches of studying root decomposition *in situ* such as root trenching and tethering roots are also discussed by Silver & Miya (2001).

Root tissue densities are often one to two orders of magnitude lower than leaf and stem material (Kramer-Walter et al., 2016). Therefore, more root material is often required to fill a litterbag. At the same time, root litter decomposes more slowly than leaf and stem litter; hence, depending on the length of study, more root litter is likely to remain in the bag at the end of any incubation period of similar duration to most leaf litter studies (e.g. one to two years). For example, in the majority of temperate grassland ecosystems with litterbags containing 0.5 g root litter, over half of the material is still likely to be present after a year of incubation. The amount of litter used in root litter decomposition studies is therefore typically lower than quantities used in leaf litter studies: anywhere between 0.2 g and 2 g of root litter (Silver & Miya, 2001; Freschet et al., 2013). Given a smaller quantity of root litter, weighing needs to be more precise, at a mimumum ± 0.001 g.

After burying the root bag in soil over a given incubation period (see next section), root litter can be extracted from litterbags, dried, and mass loss calculated as the difference between the initial dry mass and the final dry mass after incubation, similar to aboveground litter (see protocol 2.2.6 Litter decomposition). Because root litter is buried in the soil, it is more likely to be contaminated with soil, resulting in the remaining weight being higher than the initial weight. Thus, ash free corrected weight loss (i.e. the mineral residue after removal of organic matter by ignition) needs to be calculated (see protocol 2.2.6 Litter decomposition). In brief, heating undecomposed or decomposed litter in a muffle oven over 500 °C for 4 to 16 h to obtain the ash-free weight (Smith et al. 2019). Root material can also be analysed for loss of carbon and nutrients, namely total nitrogen and phosphorus, using an elemental analyser or digestion methods followed by spectrophotometry.

Installation, field operation, maintenance, interpretation

Similar to leaf litter decomposition studies, root litter should be buried underneath the same species and vegetation type as litter collection. Variation in other biological and environmental factors should be minimised in the placement of root litter. Root litter is typically buried in the soil at the main zone of root production, for example, between 5 and 20 cm below the surface in both grassland and forest sites. Samples should preferably be installed in straight lines in order to capture the same topography and facilitate the planned sequential retrieval of litterbags if carrying out multiple harvests. Once the bags have been installed, they should be marked. As they are being buried, it is ideal to tether the bag with nylon string to a metal pipe or other metallic object, for example, bottle tops. Installer's name, burial date, and GPS location of the site of litterbag placement should be recorded: a picture of the area or sketch can also be used to aid retrieval of the bags. The precise burial location can be found using a metal detector if they have been tethered to a metal object. Belowground decomposition processes may be buffered by the soil from changes and fluctuations in surface and air temperatures that potentially influence aboveground decomposition. Therefore, root-scale micro-climate should be accounted for when conducting climate change experiments. It is thus useful to bury micro-climatic sensors (temperature, soil moisture) with litterbags where possible. Alternatively, spot-measurements can be made with hand-held probes at regular intervals during the incubation period to provide information on the micro-climate.

At each sampling point, it is advised to collect a minimum of five litterbags per litter type and per site. Care should be taken when extracting buried litterbags to avoid snagging on stones, rocks, or new root growth surrounding the bag. Any holes, tears, root, and fungal growth surrounding, or in, the litterbag should be noted in the field if possible. As much soil as possible should be removed from the surface of the litterbag. Each litterbag should be placed in a separate bag and labelled. Litter should be dried at either 60 °C or 70 °C for 48 h, then the root litter removed from the mesh and root litter weight noted. Collected litter should be stored in labelled paper bags for further chemical analyses.

Where to start

Hobbie et al. (2010), Iversen et al. (2017), Löhmus & Ivask (1995), Silver & Miya (2001), Smith et al. (2014), Smith et al. (2019)

2.2.7.2 Special cases, emerging issues, and challenges

Root decomposition in anaerobic soils

Under anaerobic conditions, root litter-mass loss may be negatively correlated with increasing soil moisture. There is evidence in wetland ecosystems with organic soils that root litter may gain mass during incubation due to deposition of metallic ions, for example, the formation of iron plaques (see Weiss et al., 2005). Geochemical or Fourier-Transform Infrared (FTIR) spectroscopy can be used to correct for metal content of litter and adjust mass loss accordingly.

Global need for more root trait data

Root traits are increasingly being recognised as an important predictor of rates of decomposition (Freshcet et al., 2013; also see protocol 4.16 Functional traits). However, root trait data is lacking in comparison to leaf trait data. Currently, root trait and fine-root trait data represent only 7% and

0.03% of data, respectively, in the global trait database TRY (Kattge et al., 2011). Root data are particularly lacking from Siberia, the Middle East, north and central Africa, and Central America (Freschet et al., 2017). More root data are necessary to understand better the key traits that govern root litter decomposition. There are several morphological, chemical, and physiological root traits that can be measured as part of a root decomposition study (see Bardgett et al., 2014; Freschet et al., 2017; See et al., 2019), although the number of traits considered will add to the cost of the study.

Quantifying root litter decomposition *in situ*

Radioactive measurements have been used to estimate root age, turnover, and root C residence time (Staddon, 2004; Lukac, 2012). In order to carry out isotopic analysis, roots need to be extracted from the soil and thus have similar caveats to root litter decomposition studies. For example, Strand et al. (2008) demonstrated that poor sampling of shorter-lived smaller roots from a CO_2 enriched pine plantation resulted in an over-estimation of the longevity of root C. To date, only a few studies have combined isotope labelling and litter decomposition to address rates of root C mineralisation and stabilisation into soil organic matter. Personeni & Loiseau (2004) demonstrated the use of the technique when decomposing ^{13}C-labelled root litter underneath pot-grown living plants, yet this approach needs investigating *in situ* underneath natural communities.

Further reading

Bardgett et al. (2014), Freschet & Roumet (2017), Freschet et al. (2017), Goebel et al. (2011), Laughlin (2016), McCormack et al. (2015), See et al. (2019)

2.2.7.3 References

More on methods and existing protocols

Global reviews on root decomposition across biomes, climates, soil types, and considering various methodologies include the Fine-Root Ecology Database (FRED: http://roots.ornl.gov), Iversen et al. (2017), and Silver & Miya (2001)

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2.2.8 Soil organic matter (SOM) decomposition

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Measurement unit: carbon ha^{-1} yr^{-1}; Measurement scale: plot, site; Equipment costs: €€; Running costs: €€–€€€; Installation effort: medium; Maintenance effort: medium; Knowledge need: medium to high; Measurement mode: manual

Soil organic matter (SOM) is one of the largest terrestrial carbon pools (Schlesinger & Bernhardt, 2013) storing more than twice as much C (carbon; in the form of soil organic C: SOC) as the atmosphere (Schmidt et al., 2011). Changes in this immense stock induced by global climate change can have far-reaching effects and negative feedbacks on Earth's climate. The SOM and SOC pool size is balanced by the rate of SOM formation and by its mineralisation to inorganic carbon (CO_2), but also by losses through erosion (see protocol 2.2.10 Soil water erosion) or leaching (see protocol 2.2.9 Soil leaching) of dissolved organic carbon (DOC) (Schmidt et al., 2011; Harden et al., 2018). The World Inventory of Soil property Estimates (WISE) project WISE30s is constantly being updated and aims to calculate global soil organic stocks to a depth of 2 m, with defined uncertainty estimates (Batjes, 2016).

Climatic factors have been regarded as primary controls on SOM stabilisation and decomposition and over the resulting SOC pool in empirical and modelling approaches (Davidson & Janssens, 2006; Carvalhais et al., 2014; Luo et al., 2016). Moreover, SOM composition, its spatial or temporal accessibility for decomposer organisms (e.g. protected by occlusion, hydrophobicity, encapsulation), or its interaction with mineral surfaces can influence SOM decomposition and stabilisation (Trumbore & Zheng, 1996; Kögel-Knabner, 2002; Six et al., 2002; von Lützow et al., 2007). Importantly, SOM stabilisation and decompostion can respond differently to land management, changes in nutrient availability through fertilisation or nutrient deposition, and changing environmental conditions. It is important to increase our understanding of SOM decomposition to better project effects under future climate- and global-change scenarios (Cotrufo et al., 2015; Harden et al., 2018).

2.2.8.1 What and how to measure?

There has been a rapid development of techniques to study the soil carbon cycle. One important indicator of SOM decomposition is to determine the soil CO_2 release or soil respiration by heterotrophic organisms. For more details on soil CO_2 flux measurements see protocol 2.2.3 Soil CO_2 (and other trace gas) fluxes. Despite measuring CO_2 fluxes in the field, SOM decomposition can be estimated from short-term lab incubation by measuring CO_2 and microbial biomass and activity. Other common ways to estimate SOM decomposition is to determine SOM stocks in bulk soils (see protocol 1.4.2 Soil nutrients) or in different SOM fractions and monitor potential changes over time, and additionally to determine chemical SOM composition (such as C:N, lignin:N ratios). Stable C isotopes (^{12}C, ^{13}C) are commonly used to determine decomposition rates and turnover, and radiocarbon (^{14}C) or “bomb-carbon” dating for determining SOM-age.

Measuring CO_2 efflux of soils in lab incubations is a relatively low-cost method that can give a fast and rough overview of the microbial activity and microbe-mediated SOM decomposition. Bulk soil

SOC and ^{13}C determination by elemental analysis isotope ratio mass spectroscopy (EA-IRMS) is one of the most common and affordable methods to get a quick overview of the C stocks in the soil and requires only a small number of soil aliquots. SOM fractionation gives a very detailed overview, but is more time-consuming and needs expensive chemicals (sodium-polytungstate). Using crop cover changes to trace ^{13}C shifts in the natural abundance of C_3 and C_4 plant isotope signals over time is cheaper but requires detailed documentation of land-use changes. In contrast, using standardised ^{13}C labelled plant material as part of the SOM is more expensive, but it is better able to trace changes at finer scales. Radiocarbon dating is also a rather costly method.

Short-term lab incubations

Use compound samples of fresh soils (depending on the research question) and keep moisture content and temperature constant (depending on soil and ecosystem). Soils are incubated and CO_2 should be measured at regular intervals (e.g. weekly) and microbial biomass (see protocol 2.2.1 Soil microbial biomass - C, N and P) determined. Respiration rates can be calculated on a soil mass basis (dry soil), or per C content, or microbial C content.

C stocks, SOM fractions, and chemical composition

Bulk soil and functional SOM fractions can be analysed for C content, isotopic signature, or C age (see below). In non-calcareous soils, bulk soil C can be considered as total SOM C. In calcareous soils, samples should first be treated with 10% HCl to remove carbonates and subsequently oven dried at 60 °C for 48 h. Soil C and N content can be measured from dried and finely ground soil samples by an Elemental Analyser. SOM decomposition can be determined indirectly by monitoring net changes in C stocks in bulk soil or in different SOM fractions over time. Soil sample collection in the field and preparation are described below (see *Installation, field operation, maintenance, interpretation*).

SOM fractions can be classified to functional pools following a three-step fraction scheme to isolate their C pools based on physical, chemical, and biochemical protection mechanisms: a full description and comparison of different methods can be found in von Lützow et al. (2007). Here, we report on the methodology described by Stewart et al. (2008). This fractionation scheme is based on the assumed link between different fractions and different protection mechanisms involved in the stabilisation of organic C within the respective pools as described by Six et al. (2002). The physical fractionation of air-dried soil is conducted to obtain three size fractions: > 250 µm (coarse unprotected particulate organic matter, cPOM), 53–250 µm (microaggregate fraction: µagg), and < 53 µm (easily dispersed silt and clay: dSilt and dClay). To obtain these fractions, air-dried soil is sequentially wet-sieved through a 2 mm and 250 µm sieve to flush µagg and finer particles through the 250 µm mesh. Material that is larger than 250 µm (cPOM and sand) remains in the sieve. Microaggreagates are collected in a 53 µm sieve that is subsequently wet-sieved to separate the easily dispersed silt- and clay-sized fractions from water-stable µagg. The resulting suspension is centrifuged to separate the dSilt and dClay fractions. All collected fractions are oven dried (at 60 °C for 48 h) and weighed. The second step is the isolation of µagg. A solution of 1.85 g cm^{-3} sodium-polytungstate (SPT) is used to collect the floating fine unprotected POM (or light fraction: LF). The remaining heavy fraction is dispersed, by shaking with glass beads, and passed through a 53 µm sieve. This separates the microaggregate protected POM (> 53 µm, iPOM) and the microaggregate derived silt and clay sized fractions (µSilt and µClay). In a further step, the isolated silt and clay sized

fractions can be hydrolysed if necessary (see further description in Stewart et al., 2008). All individual fractions can be dried and used to determine C content, and for stable isotope and radiocarbon analysis (see below in section SOM age using ^{14}C (radiocarbon) dating (long-term approach).

Depending on the research question, not only SOM quantity, but also SOM quality, i.e. the chemical composition of SOM can be determined (Kögel-Knabner, 2002; von Lützow et al., 2006; Schmidt et al., 2011; Poeplau et al., 2016). Total C and total N can be measured in dried and ground soils as described above in section C stocks, SOM fractions, and chemical composition. The chemical composition of SOM can give information on the degree of plant v. secondary resources derived by decomposers and can be determined by chemolytic techniques or analytical pyrolysis in combination with solid-state ^{13}C-NMR spectroscopy or mass spectrometry on dried and ground soils (Golchin et al., 1994; Kögel-Knabner, 2002; Schmidt et al., 2011).

SOM turnover dynamics using ^{13}C isotopes (short-term approaches)

Stable isotopes, particularly the stable isotope of C (^{13}C), are a powerful means of investigating SOM decomposition. Applications range from taking advantage of changes in crop cover using natural abundance of ^{13}C, to ^{13}C labelling studies that trace ^{13}C from marked organic material into SOM and CO_2. Crop changes from C_3 plants (average δ^{13}C value of -27 ‰) to C_4 plants (e.g. maize; average δ^{13}C values of -13 ‰) or *vice versa*, will lead to a continuous modification of the ^{13}C signal of the SOM (Balesdent & Mariotti, 1996). Over time, SOM will steadily change towards the new crop ^{13}C, which allows the study of C turnover in SOM. Similarly, isotopically different litter (Djukic et al., 2013) or ^{13}C labelled plant material (leaf and/or roots) can be used to trace C sequestration into total SOM or different SOM fractions (Soong & Cotrufo, 2015). The proportion of new C incorporated into SOM (C_{store}) can be calculated as follows:

$$C_{store}(\%) = \left| \frac{(\delta^{13}C_{mix} - \delta^{13}C_{soil})}{(\delta^{13}C_{substrat} - \delta^{13}C_{soil})} \right| * 100$$

where $\delta^{13}C_{mix}$ is the δ^{13}C value of the SOM and labelled litter mix after incubation, $\delta^{13}C_{soil}$ is the initial δ^{13}C value of the soil before incubation, and $\delta^{13}C_{substrate}$ is the δ^{13}C value of the (labelled) litter used for the decomposition experiment (Djukic et al., 2013). Sample collection in natural abundance studies and installation effort of isotopically different or labelled organic matter is described below (see *Installation, field operation, maintenance, interpretation*).

SOM age using ^{14}C (radiocarbon) dating (long-term approach)

Atmospheric CO_2 contains trace amounts of the radioactive isotope ^{14}C (radiocarbon), which is produced naturally in the lower stratosphere and occurs at a relatively constant concentration in the atmosphere. Since all plants take up ^{14}C through photosynthesis they reflect the ^{14}C concentration of the atmosphere. Upon plant death the radiocarbon concentration begins to decrease through radioactive decay with a half-life of 5730 years; hence, the ^{14}C content of organic matter can serve as a measure of C age and can be translated into C residence time in a soil. Furthermore, advantage can be taken of the so called ^{14}C “bomb-carbon” peak that was caused by anthropogenic nuclear tests in the early 1960s that increased the proportion of ^{14}C in the atmosphere (Trumbore, 2009). For both approaches, both archived and recent samples are required for ^{12}C and ^{14}C analyses. Usually, accelerated mass spectroscopy (AMS) is used to measure bulk soil isotope ratios. AMS targets are

prepared according to the protocol of Rutberg et al. (1996). Samples are sealed in evacuated quartz tubes with CuO, Ag, and quartz wool and are combusted at 950 °C for 2 h. The evolved CO_2 can be used for target preparation and ^{14}C analysis by AMS.

Installation, field operation, maintenance, interpretation

Soil samples for incubations, SOM stock, and SOM fraction determination should be collected from locations within the experimental plots with homogenous vegetation. In larger experimental plots, five samples, and in smaller experimental plots, at least three samples should be collected to produce one composite sample per experimental plot replicate. Samples are collected using a soil corer or auger of known diameter. The same samples can be used to determine soil bulk density (see protocol 1.3 Soil type and physical characteristics), an essential parameter to compare C stock values among soils of different texture and composition. The upper organic layer can be collected separately and samples taken at various, but well documented soil depths. In experiments with at least two samplings, one pre-treatment (or archived sample) and one post-treatment should be analysed. Soil samples should be sieved with a 2 mm mesh and dried (e.g. at 70 °C for 48 h) in a drying oven and stored dry prior to analysis. The determination of total C and N contents and $\delta^{13}C$ and $\delta^{15}N$ contents of bulk soil samples by elemental analysis (EA) or by EA combined with isotope ratio mass spectroscopy (EA-IRMS) can provide an initial insight into the amount of SOC present in a soil and can also be used for SOM fractionation or age determination (described in section 2.2.8.1)

In tracer studies that take advantage of C_3–C_4 vegetation changes, $\delta^{13}C$ values in soil samples should be determined before and at least one year after the crop change, and changes in the isotopic composition of SOM can be used to calculate changes in SOM decomposition, see above (Balesdent & Mariotti, 1996).

Litter decomposition studies with litter differing in isotope composition (natural abundance or ^{13}C labelled material) should, depending on the litter type (see protocols 2.2.6 Foliar litter decomposition and 2.2.7 Root decomposition), place the litter on the soil surface or bury it in the soil, after determining the $\delta^{13}C$ values of the litter and of the soil. Soil litter mixes should be sampled at regular intervals (seasonal, annual), but should remain for at least 1 year and up to 3 years (depending on the ecosystem). After the soil samples have been collected, ^{13}C change and incorporation into total SOM or its various fractions over time can be determined using the methodology described above (Djukic et al., 2013; Cotrufo et al., 2015; Soong & Cotrufo, 2015).

Radiocarbon data are reported in comparison to an accepted standard value of known ^{14}C content. The most common way to express radiocarbon content of a sample is the Fraction Modern:

$$\text{Fraction Modern (FM)} = (((^{14}C / {}^{12}C)\text{sample}-25) / (0.95 * (^{14}C / {}^{12}C)\text{common standard}-19)$$

where the ratio of ^{14}C to ^{12}C has to be corrected for mass-dependent isotope fractionation to a common $\delta^{13}C$ value of -25 ‰. The denominator refers to the primary standard for reporting radiocarbon data. The correction to a common $\delta^{13}C$ value is necessary because radiocarbon, like ^{13}C, is affected by mass-dependent fractionation in the environment.

For tracking bomb-produced radiocarbon in the decades since 1963, a second nomenclature is used that reports the ^{14}C:^{12}C ratio of the sample measured in year y to an absolute standard that does not change with time, that of the oxalic acid standard decay corrected to 1950 (Trumbore, 2009):

$$\Delta^{14}C = (((^{14}C / {}^{12}C)\text{sample}-25) / (0.95 * (^{14}C / {}^{12}C)\text{common standard}-19\ e((y - 1950)/8265))) - 1) * 1000$$

For ^{14}C determination it is of overall importance to avoid any potential contamination of the samples by ^{14}C.

Where to start

Balesdent & Mariotti (1996), Cotrufo et al. (2015), Davidson & Janssens (2006), Djukic et al. (2013), Kögel-Knabner (2002), von Lützow et al. (2006), Schmidt et al. (2011), Six et al. (2002), Stewart et al. (2008), Trumbore (2009)

2.2.8.2 Special cases, emerging issues, and challenges

The guiding paradigm has been that litter mass loss corresponds to C mineralisation, neglecting that the fraction of litter that does not decay at a measurable rate (that is, mass remaining) contributes to SOM formation. Stable isotope tracing studies can be used to quantify litter-derived C and N incorporation in the mineral soil and accumulation of labile compounds and microbial products in SOM forms while mass is being lost (Cotrufo et al., 2015). A more detailed knowledge of the role of soil aggregates and of soil microbes in SOM stabilisation is needed – they are part of the SOM pool, and most of the plant-derived residues are cycled intensely within the microbial biomass (Liang et al., 2017). There is an increased notion that nutrient interactions and microbial physiology parameters should be better represented in SOC model dynamics and ecosystem models (Sierra, 2012; Cotrufo et al., 2013; Wieder, et al., 2014; Poeplau et al., 2016; Liang et al., 2017).

One of the major challenges in SOM decomposition dynamics is the spatial heterogeneity of soils. We still need a better characterisation of spatial hot-spots of SOM decomposition in soils and the rhizosphere (Herrmann et al., 2007; Mueller et al., 2013; Kaiser et al., 2015), since high labile C availability in the rhizosphere, for example, can stimulate SOM decomposition (i.e. “organic matter priming” (Fontaine et al., 2004; Kuzyakov, 2010)). We also need to characterise better the susceptibility of SOM decomposition in response to elevated CO_2, changing climatic conditions, or land-use change (e.g. Harden et al., 2018). Effects of soil meso- or micro-fauna, and soil microbial biomass on SOM decomposition, have often been overlooked in field or laboratory experiments (Djukic et al., 2013; Cotrufo et al., 2015). New fingerprint techniques, such as proteomics or metabolomics, provide a whole new toolbox to characterise SOM and SOM decomposition (Keiblinger et al., 2016).

2.2.8.3 References

Theory, significance, and large datasets

Harden et al. (2018), Melillo et al. (2017), Minasny et al. (2017), Six et al. (2000)

More on methods and existing protocols

Von Lützow et al. (2006, 2007) Stewart et al. (2008), Trumbore (2009)

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2.2.9 Soil leaching

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Measurement unit: kg ha^{-1} y^{-1}; Measurement scale: plot; Equipment costs: €€; Running costs: €€; Installation effort: medium; Maintenance effort: low; Knowledge need: medium; Measurement mode: manual

Soil leaching is the downward movement of dissolved mobile plant nutrients in the soil profile following percolating water such as rain or irrigation water. Leaching occurs when the soil pores become filled with water and water moves downward in the soil. EcologyDictionary.org defines leaching as 'The process by which soluble constituents are dissolved and filtered through the soil by a percolating fluid'. Nutrients such as nitrate, phosphorus, and base cations, as well as other constituents such as dissolved organic carbon and contaminants, are lost from the soil via leaching below the rooting zone of the vegetation. Nutrients therefore become out of the reach of plants which leads to reduced soil fertility and plant yield (Cameron et al., 2013). Leaching may further create environmental concerns when the constituents move into ground- and surface waters and oceans and cause eutrophication resulting in unwanted growth of weeds and algae. Ecosystems normally have an intra-system cycle with a closed loop recycling essential nutrients. In such a system the demand for nutrients for growth is often higher or equal to the supply of the system. However, if the supply of nutrients is higher than the demand, leaching may occur, which is often an indication of saturation and a broken nutrient cycle. Climate change will affect the distribution of precipitation and extreme events such as droughts, and thus influence the amount of nutrients lost from a system and may lead to changes in the trends of leaching. Other global change drivers, such as land-use, land-use change, or pollution can also impact soil leaching. We are still learning more about the importance and effects of such phenomena (e.g. Reichenau et al., 2016). Accurate sampling of soil solutions followed by chemical composition analysis in the laboratory provides opportunities for effectively evaluating the availability and mobility of nutrients and pollutants as an early warning of potential groundwater contamination.

2.2.9.1. What and how to measure?

Knowledge on nutrients being lost from the soil, such as through nutrient leaching below the root zone (Addiscott, 1990), is required to understand nutrient cycling. Most commonly, soil leaching is determined by two factors: i) the soil solution nutrient concentration below the root zone combined with ii) the use of hydrological models to estimate the flow of water through the soil profile. Applying these two together we may estimate the amount of nutrients leached from the system.

Soil solution sampling aims to specify the quality of soil pore water. Generally, sampling can be performed using either non-destructive or destructive methods. We recommend the use of non-destructive methods which involve the installation of an *in situ* soil solution collector. A range of non-destructive soil solution sampling techniques are available and advantages and disadvantages are compared by Litaor (1988) and later by Weihermüller et al. (2007), Fares et al. (2009), and Curley et al. (2011). The non-destructive samplers (porous cups, porous plates, capillary wicks, pan lysimeters, resin boxes, lysimeters) vary in shape, size, and chemical and physical properties.

The samplers collect water either with applied tension (suction methods) or without applied tension (zero tension). Zero-tension lysimeters are constructed from pans or PVC pipes and collect gravitational water. They generally have significantly larger collection areas than tension lysimeters and are more difficult to install causing relatively larger disturbance to the plot, especially at greater depths. Tension lysimeters, on the other hand, are normally smaller and comparatively easy to install, and they have been produced using different materials such as ceramic, glass, acrylic, porous PTFE (Teflon), and other materials which are chemically inert. During recent years Teflon lysimeters have been more frequently used. Lysimeters collect water from soil pores at specific spots and partially filter the water that enters the sampler. A system with continuous suction is preferred if a low sampling frequency is applied (monthly is recommended). The basic principle is that a constant vacuum is applied to a suction cup, which allows water to pass through. The applied suction should preferably be equivalent to the suction of the soil at field moisture capacity.

When using tension lysimeters, large soil pits do not need to be dug because lysimeters can be installed using soil cores. Tension lysimeters thus cause only negligible disturbance of the soil, especially when installed at an angle, and are cost-effective. There is no consensus as to the best techniques for soil solution collection. Tension lysimeters have been used more extensively than zero-tension lysimeters, and are currently the most universally used technique for extracting soil water.

Tension lysimeters can be installed with medium training, but the installation is rather time-consuming. The number of samplers should be based on achieving an efficient cover of the spatial variation of the specific soil. This is often compromised by the need to reduce soil disturbance that installation of the samplers causes. If possible, the number of samplers should be more than three to capture the variation between samplers.

In climate-change plot experiments it is normally impossible to install lysimeters horizontally into the soil layer of interest because disturbance would be too great. It is therefore recommended to install the lysimeters at an angle of approximately 45 degrees below the root zone depth. The lysimeters are inserted into holes of appropriate depth made by a soil auger where a slurry of non-toxic silica flour (SiO_2) has been applied. The slurry should be mixed at a ratio of approximately 5 kg silica flour to 1 L of deionized water. The mixed slurry is poured into the hole to a depth of at least 15 cm and the lysimeter unit is then placed in the auger hole pressing out the excess silica slurry. This procedure guarantees the porous surface of the lysimeter will be in close contact with the capillaries of the soil column.

Samples are evacuated using a portable pump that requires power. When the soil solution suction is less than the applied vacuum, the soil solution is drawn across the porous wall into the lysimeter by the induced pressure gradient. The soil solution sample is led from the cup by PTFE tubing to a storage collection bottle which is installed in an insulated box in order to protect the samples from temperature extremes and changes. The installation should preferably follow the manufacturer's manual. Several installation protocols are available online: a thorough guide to installing Prenart lysimeters (http://www.prenart.dk/) is found at https://lter.kbs.msu.edu/protocols/42 but it may well be used for other lysimeter types. The maintenance of the system is low. Usually, when the system is operated continuously, there are few problems. However, plugging of the sampler pores does sometimes occur and it will then be necessary to remove the samplers and flush them through. The lysimeters can be reinstalled at new sites after rinsing.

The volume of water sampled by each sampler should be recorded so that the functioning of the lysimeter may be checked. Ideally, soil solution should be sampled throughout the year but some sites will be drier than others and in dry periods water will be hard to extract and the volume of water will decrease. A large enough volume of soil water for all included measurements should be stored in plastic bottles and transported to the laboratory as fast, dark and cold as possible. The soil samples are stored in the refrigerator until they can be processed and concentrations are determined. Fast measurements are recommended to avoid any changes in the samples. To interpret the soil leaching from measurements of soil concentration, a calibrated hydrological model is used to determine the hydrological flow of water in the soil profile. Subsequently, the soil concentration is coupled to the water movement in the soil profile. Different models exist to determine the hydrological flow of water (see reviews by Pechlivanidis et al., 2011; Devia et al., 2015; Sood & Smakhtin, 2015). Such a hydrologic model is often calibrated with soil moisture data derived from using a standard method such as time domain reflectometry (TDR) or time domain transmission (TDT) as well as climate data from a nearby climate station. Also see protocol 3.1 Soil moisture.

Where to start

Addiscott (1990), Cameron et al. (2013), Fares et al. (2009), Sood & Smakhtin (2015)

2.2.9.2. Special cases, emerging issues, and challenges

Ceramic lysimeters have been criticised (Raulund-Rasmussen, 1989) because the chemical composition of the soil solution can be modified by contact with the ceramic material.

Destructive methods involve soil sampling and subsequent extraction of soil solution in the laboratory by centrifugation. Laboratory extraction of soil water is generally less time-consuming and cheaper than lysimeter installation. However, for obvious reasons, such methods involve considerable destructive disturbance to the site that often make them unsuitable for most experiments.

Plant Root Simulator (PRS®) probes are non-destructive ion exchange resins that are easily inserted into the soil. Both anion (e.g. NO_3^-, HPO_4^{2-}, SO_4^{2-}) and cation (e.g. NH_4^+, K^+, Ca^{2+}, Mg^{2+}) probes exist. These measure ion supply *in situ* in the upper soil profile and cannot be used to estimate soil leaching.

A range of studies have compared the chemical composition of soil solutions collected by zero-tension v. tension lysimeters (Haines et al., 1982; Nyberg & Fahey, 1988; Swistock et al., 1990; Hendershot & Courchesne, 1991; Marques et al., 1996). Estimates of soil solution composition and water flow differed according to lysimeter type. Zero-tension lysimeters collected more water at the upper soil depths but less water at greater depths. In general, there are no obvious patterns of divergences between lysimeter types across the various studies.

2.8.3. References

Theory, significance, and large datasets

Addiscott (1990), Cameron et al. (2013)

More on methods and existing protocols

Curley et al. (2011), Fares et al. (2009), Pechlivanidis et al. (2011), Sood & Smakhtin (2015)

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2.2.10 Soil water erosion

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Measurement unit: Mg ha^{-1} y^{-1}; Measurement scale: plot; Equipment costs: €€-€€€; Running costs: €€; Installation effort: medium; Maintenance effort: medium (frequent recording); Knowledge need: medium; Measurement mode: manual

Soil erosion can be defined as a three-phase process that consists of (i) the detachment of individual soil particles from the soil mass; (ii) their subsequent transport by an erosive agent; and, ultimately, (iii) their deposition when the erosive agent lacks sufficient energy for further transport (Morgan, 2005). In the case of soil erosion by water – the focus of this protocol – rain-splash as well as water running over the soil surface detach and then move the detached particles. Rain-splash is typically the most important detaching agent, while running water tends to be the principal transporting agent. The transport of soil particles resulting from the direct impact of falling raindrops is designated as rain-splash erosion, while the transport of soil particles by running water is commonly divided into inter-rill and rill erosion. Inter-rill erosion refers to water running as a shallow sheet ("overland flow") and removing a relatively uniform thickness of soil, whereas rill erosion refers to water running as a concentrated flow and removing soil by "gouging out" channels of increasing depth and/or width. In turn, rill erosion is generally divided into rill and gully erosion depending on channel dimensions.

Under natural stable conditions, soil systems are considered to be under steady-state conditions, as the loss of soil material by erosion from a given area (i.e. the loss of soil, its redistribution over the landscape, and its export to river systems) is approximately balanced by the formation of soil as a result of weathering (Montgomery, 2007). However, anthropogenic activities such as intensive agriculture, deforestation, urbanisation, and road construction, have increased by 10–40 times the rate at which erosion is occurring globally, causing both on-site and off-site environmental problems (Verheijen et al., 2009). On-site impacts include the loss of carbon and nutrients such as nitrogen and phosphorus from the topsoil through soil water erosion, reducing soil fertility and plant productivity, as well as threatening key soil functions, ultimately causing land degradation and desertification, as recognised by the European Commission (EC) (2006). Off-site effects include sedimentation of waterways and eutrophication of water bodies, as well as sediment-related damage to infrastructure (Cantón et al., 2011). Therefore, soil erosion is a complex process that causes the transport and deposition of sediments and associated organic carbon and nutrients within the landscape, releases CO_2 to the atmosphere due to organic matter mineralisation processes occurring during sediment detachment and transport, and preserves buried organic carbon from mineralisation in sedimentary deposits, affecting the carbon sink or source capacity of a given area and thus the global carbon cycle (van Oost et al., 2007). Moreover, and given the climate-change projections for some areas, soil erosion rates are expected to increase because high-intensity extreme rainfall and flood events are predicted to be more frequent causing significant erosion, especially in the Mediterranean Basin, but also due to other global-change drivers such as land-use changes and fires causing loss of vegetation cover and changes in topsoil properties.

As stated, soil erosion by water is the lateral movement of soil downhill following important rainfall events. Although normally overlooked, this process can be relevant for the soil carbon and nutrient

budget in low-productive ecosystems with sparse vegetation cover such as arid and semi-arid ecosystems, especially those with steep slopes and erodible soils. In addition, this process can interact with other relevant processes such as plant litter decomposition, net primary productivity, and soil carbon sequestration capacity (Lal, 2005; Throop & Archer, 2009; Almagro and Martínez-Mena, 2014). Indeed, the role of soil erosion in the terrestrial carbon budget remains one of the most important uncertainties in our attempts to determine the potential of soils to mediate climate change through carbon sequestration and stabilisation in soils (van Oost et al., 2007; Quinton et al., 2010). In this regard, there is an urgent need to adequately represent carbon and nutrients exported by erosion processes and controls in Earth System Models to gain insight into the implications for the carbon and nutrient balance at different spatial (e.g. slope, catchment, regional) and temporal (from years and decades to centuries and millennia) scales (Doetterl et al., 2016). But before this, it is necessary to accurately monitor and estimate current erosion rates and associated losses of carbon and nutrients during detachment, transported, and deposition at different spatial and temporal scales and under different climatic conditions and land uses.

2.2.10.1 What and how to measure?

As soil erosion is a very irregular process, a mixture of direct or indirect measurements, observations, and experimental evaluation of the processes is sometimes needed. Depending on the spatial (patch, hillslope, catchment) and temporal (years, centuries, millennia) scales, different approaches have to be used since different erosion and sediment transport processes have to be taken into consideration. For example, inter-rill, rill, and gully erosion can be considered as the dominant processes at the hillslope scale, but other processes such as bank erosion and landslides have to be taken into account at the catchment level. Therefore, it is important to keep in mind the specific purposes of the study for the design of suitable methods to measure the hydrological response and erosion rates, and caution should be exercised in comparing measurements based on different methodologies and temporal or spatial scales.

Traditionally, soil erosion has been measured in the field using plots of different size to determine soil loss and runoff rates under natural rainfall conditions (***gold standard***). However, if rainfall events are scarce or it is necessary to provide sufficient data to parameterise a wide array of existent soil erosion models, rainfall simulations and runoff experiments can be carried out in the plots (***bronze method***). An overview of the wide range of existing portable rainfall simulators is given by Iserloh et al. (2013).

There are different direct measurement methods depending on the spatial scale and therefore the dominant erosion process to consider.

a) Measurements of inter-rill and rill erosion rates using bounded and unbounded erosion plots

These types of plot studies remain some of the most widely used methods for estimating field erosion rates over short and medium time periods. After each rainfall event, the amount of runoff, eroded material, and carbon and nutrients transported laterally through the hillslope are estimated. The plot size depends on the ecosystem type. An appropriate plot size can be 1 m x 1 m for grasslands, 10 m x 2 m for shrublands, and 20 m x 5 m for forests and woody crops (Morgan, 2005). However, small plots (1 m^2) have the flaw of low-energy water flow for soil detachment and transport occurring at short distances, and that the high spatial variability of surface components is

underrepresented, especially in semi-arid and other patchy ecosystems (Boix-Fayos et al., 2006). As a general rule, the larger the plot, the better it will represent the heterogeneity of the system.

A bounded plot is bounded by sheets of, for example, metal which protrude 150–200 mm above the soil surface (Picture 2.2.10.1, left). It has a collecting trough or gutter at the bottom end where the runoff, with its sediment, is channelled into one or more collecting tanks, depending on runoff volumes. One advantage of bounded plots is that they allow a comparison of different responses at the same spatial scale, with exactly the same size of drainage area. However, bounded plots are not the most suitable method for long-term (> 4 years) monitoring of soil erosion rates due to the risk of exhaustion of available material within the plot (Boix-Fayos et al., 2006).

An alternative design consists of unbounded plots, avoiding edge effects and possible sediment exhaustion but introducing uncertainty about the contributing drainage area (Picture 2.2.10.1). Unbounded plots such as Gerlach troughs and sediment fences have especially been used for measuring runoff and/or sediment losses at larger spatial scales such as agricultural fields, tree plantations, or entire hillslopes, including gully erosion processes.

Given the high spatial variability of erosion patterns, the use of several replicate plots located in different locations at the bottom of a slope are encouraged. Although this type of plot requires considerable installation and maintenance efforts and event-based sampling is time-consuming, at least three replicates per field site should be set up given the high spatial variability of this response variable (Hudson, 1993). Nevertheless, it is difficult to establish a fixed number of standard replications since the number of replicates must be established individually for each study case depending on the inherent variability of each ecosystem type (Boix-Fayos et al., 2006). As a general rule, the number of replications should increase as the plot size decreases to guarantee that the spatial variability of erosion patterns is captured. In this regard, it is also very important to select contributing drainage areas that are similar in coverage and distribution patterns of vegetation, litter layer, stone pavements, and crusts before establishing adjacent replicate plots.

Picture 2.2.10.1 Bounded (left) and unbounded Gerlach-type (right) erosion plots connected to sediments and runoff collecting tanks in two study sites in Murcia (SE Spain). Photo: María Martínez-Mena (left) and Eloisa García (right)

After each erosion event, runoff and soil loss is measured by collecting the water and sediments from the storage tanks connected to the bounded or unbounded runoff plots. Sampling of the sediments from the tanks is carried out after thorough stirring taking a minimum of five aliquots of a known volume (e.g. 1 L) from different depths using plastic bottles. Along with the runoff erosion plots, a recording rain gauge in the experimental area is desirable to measure precipitation after each event.

Both sediment and water samples should be ideally stored in a portable fridge at 4 °C during their transport from field to laboratory. Later in the laboratory, the sediment collected in the sediment traps and the runoff are analysed for total suspended solids (TSS), organic carbon, and nutrients. Ideally, samples should be processed immediately after collection, but if not possible, samples should be stored at 4 °C for short periods or frozen (-20 °C) for long periods. Annual losses by erosion (in g m^{-2} yr^{-1}) are calculated as the sum of the concentration of each parameter (TSS, carbon, nutrients) measured in each erosion event during one year, divided by the surface of the bounded plots or by the known contributing drainage area of each sediment trap (that can be estimated from a Digital Terrain Model with a certain resolution).

b) Measurements of erosion processes occurring at larger spatial scales (i.e. slope and catchment)

Measurements of erosion processes at larger spatial scales such as gully erosion and mass movements can also be monitored in the field. A simple method of estimating cumulative volume of soil removed by rill or gully erosion on a slope is to determine the cross-sectional area of the rills/gullies along a series of transects of 20–100 m long across the slope (see Stocking & Murnaghan (2001) and Morgan (2005) for details on the protocols). The bulk density (see protocol 1.3 Soil type and physical characteristics) of the removed soil is then needed to estimate the sediment losses by weight. A similar approach can be used to estimate the volume and weight of sediments recently deposited on hillslopes or at footslopes, measuring their length, width, depth, and bulk density.

For mass movements (bank erosion, landslides) soil erosion can be estimated using tracers either for the short- (^{7}Be) or long-term (^{137}Cs and ^{14}C). The use of these radioactive isotope tracers in soil profiles can provide not only qualitative information on the patterns of soil erosion/deposition in a landscape over time depending on the decay rate of the isotope, but also estimates of soil erosion rates when combined with conversion models such as the proportional approach or the mass balance model (Morgan, 2005).

At the catchment scale, given the episodic nature of water erosion processes, continuous and long-term monitoring of sediment fluxes is desirable. However, these more integrative measurements are costly and time-consuming, and hence mostly focus on measurements at the catchment outlet. In this regard, an inexpensive and reliable means of automatically collecting water samples for suspended-sediment measurements in ephemeral channels and headwater streams is the siphon sampler (Diehl, 2008). Multiple samplers are spaced vertically to collect suspended-sediments samples at different depths (Picture 2.2.10.2). Each sampler includes a sample container (typically a 0.5 or 1 L bottle), an intake tube, and an air vent. The siphon samplers are useful when multiple and/or remote sites need to be sampled. However, some limitations of this sampler are that the time at which the sample is taken is unknown, the stage at which sampling begins depends on the intake orientation and on the stream velocity and turbulence, and the sand concentration contained in the sampler can be higher or lower than that of the stream. It is very important to select an appropriate location to install the sampler to get a representative sample without compromising its functionality. For example, if the sampler is installed in the main channel it can be exposed to debris impact and accumulation as well as strong hydraulic forces that can affect representativeness of the sample. For more details on pros and cons and on how to build a siphon sampler see Diehl (2008).

Picture 2.2.10.2 Siphon samplers placed in an intermittent (left) and ephemeral (right) stream in a semi-arid catchment in Murcia (SE Spain). Multiple samplers are spaced vertically to collect suspended-sediments samples at different depths during a flooding event. Photo: María Almagro.

Where to start

Boardman & Poesen (2007), Diehl (2008), Hudson (1993), Lal (1994), Morgan (2005), Stocking & Murnaghan (2001), Wischmeier & Smith (1978)

2.2.10.2 Special cases, emerging issues, and challenges

Although this protocol focuses on direct field measurements of soil water erosion at different spatial scales, including different forms and types of erosion plots, morphological transects, and siphon samplers, there are many other direct methods such as erosion pins, quantification with ^{137}Cs, experimental catchments, and bathymetrical surveys of reservoirs, as well as several indirect methods to monitor runoff and soil erosion, such as modelling, remote sensing and GIS analysis, and estimation with topographic benchmarks related to vegetation, dendrochronology, and digital elevation model (DEM) reconstruction. For more details, see Cantón et al. (2011) and Doetterl et al. (2016).

Emerging approaches to obtain the spatial distribution of soil erosion and deposition processes consist of large-scale terrestrial and aerial monitoring using drones and digital photogrammetry (Hackney & Clayton, 2015).

2.2.10.3 References

Theory, significance, and large datasets

Boardman (2006), Boardman & Poesen (2007), Boix-Fayos et al. (2006, 2007), Maetens et al. (2012), Quinton et al. (2010), Renschler & Harbor (2002), van Oost et al. (2007), Vanmaercke et al. (2011, 2012), de Vente & Poesen (2005)

More on methods and existing protocols

Doetterl et al. (2016), European Commission (2006), Iserloh et al. (2013), Hackney and Clayton (2015)

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2.2.11. Biological nitrogen fixation

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Measurement unit: N ha^{-1} y^{-1}; Measurement scale: plot; Equipment costs: none; Running costs: €€–€€€; Installation effort: medium; Maintenance effort: medium; Knowledge need: medium; Measurement mode: manual

Biological nitrogen fixation (BNF) is the largest natural source of exogenous nitrogen (N) in unmanaged ecosystems (Vitousek et al., 2013) and also the primary baseline against which anthropogenic changes to the N cycle are measured (Vitousek et al., 1997). BNF arises from both symbiotic associations in the form of root nodules between bacteria and plants as well as free-living microorganisms (e.g. in leaf litter and soil), called diazotrophs (Reed et al., 2011). Broad-scale estimates of BNF in natural temperate ecosystems, especially in late-successional temperate grasslands and forests, are consistently low, while the highest rates of naturally occurring BNF presumably occur in the evergreen lowland tropical rainforest (Cleveland et al., 1999; Vitousek et al., 2013). Low estimates of BNF in temperate grasslands and forests are mainly due to a scarcity of symbiotically fixing plant species in these ecosystems. Recently, however, Moyes et al., (2016) found diazotrophs living inside pine tree needles, providing an understudied source of N to temperate and boreal pine forests. In agricultural systems, crop legumes that symbiotically fix atmospheric N are commonly used as a renewable source of N in agriculture and their N-fixing capacity depends on microbial, soil, and environmental variables (Peoples et al., 1995). Tropical rainforests contain a high abundance and diversity of putative symbiotically fixing plant species and studies have shown that tree nodulation, and by extension symbiotic N fixation, is influenced by forest maturity and disturbance (Barron et al., 2011; Bauters et al., 2016). Barron et al. (2011) concluded that the observed pattern is evidence of the suspected inverse relationship between soil nitrate levels and nodulation, as consistent with feedback control by local N availability. There appears to be a relationship between phosphorus (P), molybdenum (Mo), and free-living BNF (Wurzburger et al., 2012; Reed et al., 2013), where BNF is constrained by P, Mo, or both. The interplay between these two elements in the organic soil layer has been put forward as an important determinant of free-living BNF rates in various neotropical forests (Wurzburger et al., 2012; Reed et al., 2013), temperate grasslands, temperate forests (Jean et al., 2013), and boreal forests (Rousk et al., 2017a). The determination of ecosystem-wide rates and controls of BNF is crucial to placing anthropogenic changes to the N cycle in context (Vitousek et al., 2013). The large-scale effects of a changing climate on BNF remain unknown, as there are only a few studies that have measured BNF after temperature and/or moisture manipulation. However, a forest inventory study suggests that N-fixing tree abundance will increase with climate warming (Liao et al., 2017), and a study on boreal mosses reported N fixation increases in response to higher temperatures (Rousk et al., 2017b)

2.2.11.1 What and how to measure?

Gold standard

The most accurate determination of N fixed over a given time period is a direct measurement through the use of isotopically labelled N: $^{15}N_2$ gas, called the **^{15}N tracer method**. First a sample is

taken in the field. This can be a soil sample (preferably topsoil as N_2 fixation rates decline rapidly with depth), vegetation sample (fresh leaf, fallen litter) or roots with nodules. Typically, samples are small (+- 25 g for soil or a few leaves or roots) and will immediately be placed in the incubation chamber. On the day of sampling, the chamber is enriched with $^{15}N_2$ and samples are allowed to fixate during a predetermined time period. Initiation of the incubation should be done as fast as possible because most research questions concern in vivo rates of N_2 fixation and thus changes in microbial activity should be avoided. After incubation, the sample is dried, ground, and analysed with a mass spectrometer to determine the ratio between ^{14}N and ^{15}N on a weight basis (Furnkranz et al., 2008). This ratio is then compared to the ^{14}N / ^{15}N ratio of a sample blank, which was incubated in regular air in parallel, and used to calculate the amount of N that was fixed during the incubation period. Once the sample is dried, it can be stored for several months as long as there is no contact with other ^{15}N sources. The main advantages of this method are that it is a direct measurement of N fixation and it is the only technique that unequivocally proves N fixation. For chamber enrichment, the highest quality of ^{15}N should be used, so at least 98 % ^{15}N. Unfortunately, this gas is very expensive, as is the required isotope-ratio mass spectrometer that measures the small increases in the ^{15}N / ^{14}N ratio, making the method unsuitable for the incubation of large volumes (Unkovich et al., 2008).

Bronze method

The enzyme nitrogenase, responsible for the reduction of N_2 into ammonia (NH_3) in diazotrophs, is also capable of reducing acetylene (C_2H_2) into ethylene (C_2H_4) (Hardy et al., 1968). Thus, acetylene can be used as an alternative substrate to N_2, in a method called the **Acetylene Reduction Assay** (ARA; Hardy et al., 1968). This method is suitable for similar sample types as the ^{15}N tracer method. On the day of sampling, samples are placed in an airtight chamber or cuvette and exposed to a C_2H_2 enriched atmosphere (usually 10 % C_2H_2 in air) for the duration of the incubation. Incubation in an C_2H_2 enriched atmosphere can be done immediately in the field or after transportation in the lab. The incubation time is seen as a trade-off between ethylene accumulation and isolation within a closed-off environment. Typically, samples with high fixation rates, such as root nodules, are incubated for anywhere between 30 min to a couple of hours (e.g. Menge & Hedin, 2009), while samples known to possess lower fixation rates, such as tropical topsoil or boreal bryophytes, are incubated for 18–24 h (e.g. Černá et al., 2009; Rousk & Michelsen, 2016). The rate of C_2H_4 accumulation in gas samples collected over a period of time is measured by a gas chromatograph (Vessey, 1994). Gas samples are recommended to be analysed as quickly as possible to avoid problems with leaking sampling vials. Given that acetylene is cheap and easy to make (if so desired) in the lab, and the technique is not very labour intensive, many measurements can be undertaken on a daily basis. The sensitivity of this method to detect nitrogenase activity is unparalleled, yet it does not directly measure N fixation and relies on the conversion of produced C_2H_2 into N fixed for quantification of N_2 fixation. While there is theoretical and empirical support for a conversion ratio of C_2H_4 produced to N_2 fixed of 3–4 to 1 (Nohrstedt et al., 1983), C_2H_2 reduction assays should ideally be calibrated by means of $^{15}N_2$. Lastly, there is also evidence for a decline in nitrogenase activity after exposure to C_2H_2 in some legume species (Hunt & Layzell, 1993), casting even more doubt upon the quantitative aspects of the assay.

Installation, field operation, maintenance, interpretation

The operation in field and lab is similar for free-living and symbiotic BNF and besides the sensitive measuring equipment, such as the gas chromatograph and mass spectrometer, no maintenance is required.

Symbiotic BNF

Depending on your research question, root nodules are excavated and can be (i) incubated either detached from roots or (ii) the root system can be incubated entirely (Peoples et al., 2009). The first will yield a fixation rate per gram of nodule (g N fixed g^{-1} h^{-1}) and will need a complementary measurement of the nodule density (g $plant^{-1}$) and N concentration per plant along with knowledge of the amount of plants per plot to be able to upscale the results. The second is not applicable for trees, but in the case of small crop legumes it gives a good estimate of N fixation at the plot level (kg N ha^{-1} y^{-1}), provided the biomass of crop legume roots (kg ha^{-1}) is known. The estimation of plant dry matter, N concentration, and nodule density represent a considerable source of error in field estimates.

Free-living BNF

A sample from the substrate on which BNF is to be measured is incubated. Since free-living BNF can be very patchy and so called “hotspots” (Alexander & Schell, 1973; Pérez et al., 2008; Reed et al., 2010) with higher fixation rates than the surrounding areas have been observed, it is paramount to have a sampling strategy that covers the desired plot well. The resulting fixation rate will typically be expressed in N fixed per gram of substrate per time unit (mg N g^{-1} h^{-1}) in the case of soil or litter or expressed in N fixed per area per time unit (mg N cm^{-2} h^{-1}) in the case of tree phyllosphere (Furnkranz et al., 2008). Usually, this value is up-scaled and reported for the plot level (kg N ha^{-1} y^{-1}) through knowledge of soil density (kg m^{-3}) and litter density (kg m^{-2}). Upscaling the phyllosphere fixation rates is harder, since knowledge about the leaf surface per tree (m^2 $tree^{-1}$) and trees per plot (tree ha^{-1}) is required. Plant trait databases, such as TRY (Kattge et al., 2011), can help find this information.

It is important to keep in mind that fixation hotspots cause large errors in up-scaling and the free-living fixation rate often displays strong seasonal variation (Reed et al., 2007; Pérez et al., 2008), making several samplings per year to capture all seasons a necessity.

Where to start

Furnkranz et al. (2008), Hardy et al. (1968), Herridge et al. (2008), Reed et al. (2011), Unkovich et al. (2008)

2.2.11.2 Special cases, emerging issues, and challenges

One of the main challenges in nitrogen fixation studies is quantifying N_2 fixation in environments where fixation is expected to be low or sporadic, such as in soils. Using the acetylene assay in these environments may not produce detectable ethylene concentrations as production rates are low and the incubation time short. Prolonging the incubation time may help solve this problem. However, it may also introduce possibly unwanted side effects, such as the initiation of the anaerobic metabolism or decrease of the moisture content, due to prolonged isolation of the sample from the

environment (O'Toole & Knowles, 1973). In these instances it may be wise to replace the acetylene assay with the ^{15}N tracer technique, as it is more sensitive, although this does not always result in detectable fixation rates. In soils in particular, background N pools (primarily stemming from organic matter) may be large and the increase in ^{15}N:^{14}N ratio unnoticeable compared to this background pool (Angel et al., 2018).

An emerging technique of bypassing the diluting effects of a large background N pool in soils is to measure the ^{15}N enrichment in specific microbial biomolecules, such as DNA, RNA, and proteins (Angel at al., 2018). Increases in ^{15}N within any of these molecules would indicate N_2 fixation and, provided an increase in DNA is detected, growth. As it stands, the main challenges for this technique will be i) application, as it is not generally applicable across diverse microbial species, ii) up-scaling, as the technique requires a great amount of time to perform, limiting the amount of samples that can be processed, and iii) costs, as $^{15}N_2$ tracer gas is expensive and access to an isotope ratio mass spectrometer and a microbial laboratory is needed for all manipulations involving DNA, RNA, and proteins. In spite of its drawbacks, the application of this technique has the potential of studying free-living diazotrophs in environments where it was thus far nearly impossible.

2.2.11.3 References

Theory, significance, and large datasets

We recommend Reed et al. (2011) and Herridge et al. (2008) for information on biological N fixation in natural ecosystems and agricultural systems, respectively.

More on methods and existing protocols

For more detailed information on different protocols to measure N fixation we recommend Unkovich et al. (2008). Furnkranz et al. (2008) describes how to measure N fixation using the ^{15}N isotopic method and the report by Hardy et al. (1968) provides detailed benefits and drawbacks of the acetylene reduction assay.

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2.3 Ecosystem

2.3.1. Ecosystem CO_2 and trace gas fluxes

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Measurement unit: carbon per area and time; Measurement scale: plot; Equipment costs: €€–€€€; Running costs: €–€€; Installation effort: medium to high; Maintenance effort: low to high; Knowledge need: medium to high; Measurement mode: manual or data logger

The exchange of CO_2 between terrestrial ecosystems and the atmosphere is a key aspect of the climate system as it determines the capacity of ecosystems to sequester anthropogenic CO_2 emissions. Moreover, gas exchange measurements offer a non-destructive way to follow the system's response to environmental changes. Net CO_2 exchange (NEE) is the difference between plant CO_2 uptake (GPP) and ecosystem respiration (ER). The latter combines plant and microbial respiration. All these processes are sensitive to environmental changes such as increasing atmospheric CO_2, climate change, nitrogen deposition, and biodiversity loss (e.g. De Boeck et al., 2007; Vicca et al., 2007; Schmitt et al., 2010; Xu et al., 2016; Ryan et al., 2017; Nogueira et al., 2019). The combined responses will determine to what extent terrestrial ecosystems remain a carbon sink or become a source of carbon to the atmosphere.

Besides CO_2, plants and soil exchange trace gases such as CH_4, N_2O, and biogenic volatile organic compounds (BVOCs, for example: methanol and isoprene; see protocol 5.14 BVOCs, emissions from plants and soils) with the atmosphere. Whereas CH_4 and N_2O are greenhouse gases with a high global warming potential, isoprene and monoterpene emissions play major roles in the ecological relationships among plants and between plants and herbivores (Peñuelas et al., 2013) as well as in climate (e.g. influencing cloud formation; Zhao et al., 2017).

2.3.1.1. What and how to measure?

In experiments with short vegetation (e.g. grassland, heathland), ecosystem CO_2 and trace gas fluxes are typically measured using a closed system, including soil and plants. For CO_2, GPP and ER can be distinguished by combining measurements in the light and in the dark (by using a dark chamber or covering the transparent chamber with a cloth). The CO_2 flux measured from a transparent chamber is NEE, the CO_2 flux from a darkened chamber provides an estimate of ER. GPP can then be calculated as the difference between NEE and ER.

A transparent chamber (often custom-built) is fitted on pre-installed collars (e.g. a collar equipped with a rubber sealing or a water-filled groove; Pumpanen et al., 2004) to ensure airtight sealing. If the soil, roots, and water flow are to remain intact one can connect a windshield to the bottom of the chamber and weigh it down with a heavy chain to ensure a closed system. These chambers also require one or more fans to improve air mixing. It is important to ensure that the air inside the chamber is neither pressurised nor underpressured, as a pressure difference will create an advective flux through the soil underneath the chamber edge (Pumpanen et al., 2004). The chamber should be equipped with a sensor to measure light intensity or photosynthetically active radiation (PAR) and a temperature sensor to measure air temperature within the chamber. The chamber is connected to,

for example, an infra-red gas analyser to measure the changes in the CO_2 concentration continuously during the measurement period.

Ecosystem flux measurements in forest experiments are more challenging because of the size of the trees. Eddy covariance is rarely an option as plots are much smaller than the footprint. Ecosystem CO_2 fluxes in forests are therefore typically derived by upscaling leaf, branch, stem, and soil flux measurements (e.g. Tang et al., 2008; see protocols 2.1.4 Plant respiration and 2.3.3 Upscaling from the plot scale to the ecosystem and beyond), although whole-tree chambers have also been used (e.g. Medhurst et al., 2006).

Where to start

De Boeck et al. (2007), Medhurst et al. (2006), Xu et al. (2016)

Installation, field operation

Chambers are typically placed on permanently installed collars. Because collar installation may disturb the gas fluxes, some equilibration time needs to be allowed (a few days should suffice, but this can be monitored). Collar insertion depth should be as shallow as possible to avoid root cutting (Wang et al., 2005); 1–2 cm insertion depth is sufficient to gain a sealing effect between soil and collar/chamber.

Chambers are typically closed for only a few minutes. In case of very small fluxes, however, closure time may need to be lengthened to ensure robust flux calculation. In that case, extra caution is required to ensure conditions inside the cuvettes (especially temperature and humidity) do not change significantly over the time of the closure.

See also protocols 2.1.4 Plant respiration and 2.2.3 Soil CO_2 (and other trace gas) fluxes for further information regarding field operation.

Interpretation

While GPP reflects only plant processes, ER combines plant and microbial respiration. When the primary interest is in plant processes, aboveground (plant) respiration (R_{above}) can be estimated by combining measurements of ER and soil CO_2 efflux (see Protocol 2.2.3 Soil CO_2 (and other trace gas) fluxes); R_{above} is the difference between ER and soil CO_2 efflux (see e.g. de boeck et al., 2007). Ecosystem CO_2 fluxes depend strongly on the environmental conditions: GPP is strongly driven by light, while respiration is primarily related to temperature. In order to usefully compare these fluxes between plots, light response curves are fitted to the GPP data and and temperature response curves are fitted to the ER and R_{above} data. Commonly used equations are provided in, for instance, Casella & Soussana (1997) and De Boeck et al. (2007). Subsequently, ecosystem CO_2 fluxes can be compared at standard conditions. Depending on the research question, normalisation for plant cover or biomass may be desirable (see e.g. Vicca et al., 2007; see protocol 2.1.1 Aboveground plant biomass). Depending on the aim of the study, one may need to take into account that leaf respiration is partially inhibited in the light, and deriving GPP from NEE and ER measurements may result in a 5–10% overestimation of GPP (Atkin et al., 2000).

2.3.1.2 Special cases, emerging issues, and challenges

In recent years, advanced infrastructures – so-called ecotrons – have been developed in which ecosystem gas fluxes can be measured continuously while at the same time allowing precise conditioning of environmental factors such as temperature, relative humidity, precipitation, and CO_2 concentration (see e.g. Roy et al., 2016). More of such infrastructures will be developed in the near future within the European infrastructure network ANAEE.

2.3.1.3 References

Theory, significance, and large datasets

Kutzbach et al. (2007), Lu et al. (2013), Schmitt et al. (2010)

More on methods and existing protocols

Kutzbach et al. (2007), Wohlfart et al. (2005)

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2.3.2. Total belowground carbon flux

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Measurement unit: g C m^{-2} y^{-1}, Mg C ha^{-2} y^{-1}; Measurement scale: plot; Installation costs: €€€; Running costs: €€€; Installation effort: high; Maintenance effort: medium; Knowledge need: high; Measurement mode: manual or data logger

The total belowground carbon flux (TBCF) describes the flow of carbon from photosynthetic organisms through the phloem to the belowground system. It is critical to recognise that aboveground litterfall is not included in TBCF (Davidson et al., 2002). As such, it provides an estimate of belowground "allocation" or, if divided by gross primary production (GPP), of belowground partitioning (Litton et al., 2007). This carbon flow supports root biomass production, root respiration, root exudation, and the many heterotrophic organisms that rely on recent photosynthate for their respiratory substrate. Although it has been quantified in rather few studies so far, it is likely to be influenced by climate change and other global-change drivers, because temperature (Litton & Giardina, 2008), CO_2 (Palmroth et al., 2006), and nitrogen deposition (Liu & Greaver, 2010) all have an effect on aboveground sources and belowground sinks of carbon. It is critical to recognise that this is an estimation of the flux, not the accumulation of biomass belowground: the two may be completely uncoupled (Litton et al., 2007). The method has so far been applied mostly to forests, but it has also been applied to CO_2 effects on grasslands (Adair et al., 2009) and grass species differ in belowground flux (Sumiyoshi et al., 2017). The measurement is based on a soil carbon budget using component processes described in protocols 2.1.2 Belowground biomass, 2.2.3 Soil CO_2 (and other trace gas) fluxes. Specifically,

$$TBCF = R_s + \Delta SOM + \Delta CR - LF$$

where R_s is cumulative annual soil respiration, ΔSOM is the annual increase in soil organic matter, ΔCR is the annual increase in coarse root biomass, and LF is annual aboveground litterfall and subtracted in the equiation (see 2.1.1 Aboveground plant biomass for how to measure litterfall). Although estimates of these parameters are not rare, they have seldom been combined to estimate TBCF, which has limited the application of this concept to date.

2.3.2.1 What to measure?

Gold standard

Some of the components in the equation above have been discussed elsewhere in this chapter (see above), except perhaps coarse-root biomass increase in trees. This can be estimated using allometric equations based on stem diameter (McDowell et al., 2001). Note that ΔCR is positive when stem diameter increases.

Bronze standard

It is often true that ΔSOM is stable relative to the other carbon fluxes, at least in the absence of disturbance or nitrogen fertilisation. Under such conditions, it may be justified to set this term to

zero (Litton et al., 2007) or to some low value (e.g. 5 g C m^{-2} yr^{-1}; Peltoniemiet al., 2004). This assumption is likely invalid in manipulation experiments. This measurement relies on measurements made using other techniques and described elsewhere in this manual (see above).

2.3.2.3 References

Theory, significance, and large datasets

Raich & Nadelhoffer (1989), Davidson et al. (2002), and Giardina et al. (2014) provide good overviews of the method and global summaries of TBCF estimates from forests. Gill & Finzi (2016) have presented a meta-analysis of data by biome.

More on methods and existing protocols

Litton et al. (2007) provide a careful discussion of terminology and a comparison among intensively monitored sites. Where necessary, one should also account for the effects of disturbance (Sumiyoshi et al., 2017) and nitrogen fertilisation on ΔSOM (Janssens et al., 2010). Finally, because this method is based on a difference between sums rather than a direct measurement, error propagation must be considered.

All references

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2.3.3 Upscaling from the plot scale to the ecosystem and beyond

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Measurement unit: Mg C ha^{-1} yr^{-1} or Mg C ha^{-1}; Measurement scale: point, plot, ecosystem; Installation costs: €–€€€; Running costs: Not applicable; Installation effort: medium; Maintenance effort: medium; Knowledge need: medium; Measurement mode: manual or data logger

As climate change progresses, increasing frequency and severity of extreme events as well as gradual changes in precipitation and temperature regimes threaten terrestrial carbon (C) sinks, making it increasingly important to study ecosystem C stock and C exchange responses to these events (Schwalm et al., 2012; IPCC, 2014). Coupling upscaling techniques with field measurements allows for the extrapolation of biomass, ecosystem C exchange, and photosynthesis data to larger sample areas, eliminating the need for extra time and resources to be spent on collecting additional field data, while also providing a method to monitor less accessible locations (Devagiri et al., 2013; Asner & Mascaro, 2014). The upscaling methods suggested in this protocol can be easily applied to other climate-change drivers as well as any type of ecosystem study that involves measurements at plot scale with broader scale implications. The method in upscaling suggested here applies particularly to ecosystem scale observations such as ecosystem C uptake (ecosystem scale photosynthesis or GPP; ecosystem net primary production or NPP), ecosystem C release (ecosystem respiration or Reco), and net ecosystem C exchange (net ecosystem exchange of C or NEE).

2.3.3.1 What and how to measure?

Upscaling carbon exchange

Ecosystem CO_2 fluxes are most commonly quantified by either the biometric method or the eddy covariance method (see Campioli et al., 2016 and references within for comparison between the two methods). Biometric methods upscale growth and CO_2 flux measurements performed on individual plants or small plots to ecosystem carbon exchange (Cresto Aleina et al., 2016; denoted g C m^{-2} y^{-1} but more often denoted as mg CO_2 – C m^{-2} s^{-1}). This is particularly relevant for forests where ecosystem scale flux measurements are only possible with the eddy covariance technique, but the large footprint of the latter usually precludes its use in manipulation experiments (but see e.g. Gough et al., 2013).

Upscaling methods can be classified to data-driven and data-assimilation approaches (see Xiao et al., 2011 and references within). Data-driven approaches are based on empirical, statistical models and are trained with flux observations and various explanatory variables such as land cover, enhanced vegetation index (EVI), photosynthetically active radiation (PAR), land surface temperature, and land surface structure. The empirical models are often a set of multivariate linear submodels under various conditions (rule-based models). Data-assimilation approaches based on simple ecosystem models and parameter estimation techniques, where measurements are used to optimise the parameters of the models, and the optimised models are then used for the estimation of fluxes over broad regions. The Markov chain Monte Carlo (MCMC) and differential evolution (DE) methods can be used in parameter optimisation.

For how to measure biomass, photosynthesis, and carbon fluxes see protocols 2.1.1 Aboveground plant biomass, 2.1.2 Belowground plant biomass, 2.1.3 Leaf-scale photosynthesis and 2.3.1 Ecosystem CO_2 and trace gas fluxes.

Measurement unit: Mg C ha^{-1} yr^{-1}; Equipment costs: €€–€€€; Running costs: not applicable; Installation effort: medium; Maintenance effort: medium; Knowledge: medium; Measurement mode: logger or manual

Where to start

Campioli et al. (2016), Cresto Aleina et al. (2016), Desai (2010), Lee et al. (2011), Sun et al. (2011)

Upscaling biomass

Gold standard

Lidar, a tool that uses light detection and ranging, is the premier instrument used to quantify and upscale carbon stocks, in part due to vertical height detection (a metric highly correlated with biomass in forested ecosystems) (Chen, 2013). Lidar data are collected via airborne flights (UAV fixed-wing aircrafts) or satellite (upcoming GEDI mission by NASA). To estimate aboveground biomass, height measurements are extracted from the lidar point cloud, paired with ground measurements, and modelled across the whole coverage area (Patenaude et al., 2004). This technique is expensive and airborne lidar surveys are only done on-demand; however, in late 2018, NASA launched GEDI (Global Ecosystem Dynamics Investigation Lidar), a satellite projected to take 15 billion cloud-free observations globally over 24 months. One of their goals is to quantify aboveground biomass, and carbon stocks and flows of forest using a globally consistent data. If resources are limited, NDVI (normalised differential vegetation index) and SAR (synthetic aperture radar) can be extracted from satellite multispectral optical data (see *Bronze standard*).

Bronze standard

While some studies upscale using national or continental inventory data (provided by organisations such as the Food and Agriculture Organization of the United Nations (FAO), the Forest Inventory and Analysis (FIA), or the US Department of Agriculture (USDA)), these estimates can exhibit considerable temporal and spatial variation and uncertainty. If lidar data are unobtainable, other remote sensing techniques are available for biomass upscaling including NDVI and SAR (see Devagiri et al., 2013; Laurin et al., 2017). These data products are openly available and can be accessed using NASA's MODIS (Moderate Resolution Imaging Spectroradiometer) website.

Measurement unit: Mg C ha^{-1}; Equipment costs: €–€€€; Running costs: not applicable; Installation effort: medium; Maintenance effort: medium; Knowledge: medium; Measurement mode: logger or manual

Where to start

Asner & Mascaro (2014), Chen (2013), Devagiri et al. (2013), Laurin et al. (2017), Patenaude et al. (2004)

Upscaling photosynthesis

Gold standard

Two main process-based model types are used for upscaling photosynthesis to ecosystem level: "big leaf" models and two-leaf models. The "big leaf" principle (Farquhar et al., 1980, Farquhar & von Caemmerer, 1982) is most commonly used in ecosystem models and assumes that canopy photosynthesis responds similarly to a single unshaded canopy leaf, where chloroplasts acclimate to the within-leaf light profile. However, total canopy photosynthesis often varies from up-scaled single-leaf derivations due to canopy light level complexity caused by sun flecks, leaf angle variation, and canopy clumping (Kull, 2002). This underestimation becomes especially evident in structurally complex systems (such as forests). The two-leaf models divide canopies into sunlit and shaded sections, or in more complex models, divide canopies into several layers and incorporate leaf angle (de Pury & Farquhar, 1997). Two-leaf models appear to represent photosynthesis more adequately in more complex environments (Mercado et al., 2009; Sprintsin et al., 2012).

Measuring large-scale photosynthesis as gross primary production (GPP):

1. In more simple ecosystems (grasslands, shrublands), canopy gas-exchange chambers can be used for estimations of photosynthesis (see protocol 3.3.1 Ecosystem CO_2 and trace gas fluxes).
2. For more complex systems, such as forests, eddy-covariance data can be used (see protocol 3.3.1 Ecosystem CO_2 and trace gas fluxes) but this cannot be used for small treatment plots.
3. A third possibility is to measure canopy reflectance from above the plant canopy (see protocol 5.12 Reflectance assessments of plant physiological status). Different reflectance indices, such as PRI and others have been shown to correlate with leaf-level photochemical efficiency of plants (Gamon et al., 1992; Garbulsky et al., 2013), however ecosystem structure strongly affects the use of reflectance indices for photosynthesis estimations (Mänd et al., 2010; Vicca et al., 2016). For larger areas, it might be possible to use already available reflectance data (e.g. data acquired by satellite-born MODerate resolution Imaging Spectrometer – MODIS, NASA), but this is not possible for small plots.

Bronze standard

If direct measurements of leaf photosynthetic capacity are not possible, databases of species-specific maximal photosynthetic capacity can be used for model parametrisation (https://daac.ornl.gov/VEGETATION/guides/Leaf_Photosynthesis_Traits.html), but it should be noted that photosynthesis is likely to be altered in climate-manipulation experiments.

Where to start

Farquhar et al. (1980), Sprintsin et al. (2012)

2.3.3.3 References

Theory, significance, and large datasets

Farquhar & von Caemmerer (1982), Jung et al. (2011), Xiao et al. (2011)

More on methods and existing protocols

Asner & Mascaro (2014), Campiol et al. (2016), Jung et al. (2011), Xiao et al. (2011)

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