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Abstract. The nutritional status of plant canopies in terms of nutrients (C, N, P, K, Ca, Mg, Mn, Fe, Cu, Zn) exerts a strong influence on the carbon cycle and energy balance of terrestrial ecosystems. Therefore, in order to account for the spatial and temporal variations in nutritional status of the plant species composing the canopy, we detail the methodology applied to achieve consistent time-series of leaf mass to area ratio and nutrient content of the foliage within the footprint of the Integrated Carbon Observation System Ecosystem stations. The guidelines and definitions apply to most terrestrial ecosystems.

Keywords: ICOS, foliage nutrient content, protocol, leaf mass-to-area ratio

INTRODUCTION

The nutritional status of the plant canopies in terms of macro- and micronutrients (C, N, P, K, Ca, Mg, Mn, Fe, Cu, Zn) has a strong influence on the carbon and nitrogen cycles as well as the energy balance of terrestrial ecosystems (Fernandez-Martinez *et al.*, 2014; Jonard *et al.*, 2009, Ollinger *et al.*, 2008). The leaf mass-to-area ratio (*LMA*, kg dry mass × m^{-2} leaf area) and nutrient content of the foliage may determine the spatial and temporal variations of canopy characteristics, such as the spectral reflectance, albedo, photosynthetic capacity or temperature independent respiration rate per unit foliage area, that are key variables with respect to the objective of the pan-European research infrastructure Integrated Carbon Observation System (ICOS). These characteristics control to a large extent the ecosystem radiative and energy balances (Dillen *et al.*, 2012; Hollinger *et al.*, 2010; Jacquemoud *et al.*, 2009; Knyazikhin *et al.*, 2013), canopy photosynthesis (Delzon *et al.*, 2005; Fleischer *et al.*, 2013; Leuning *et al.*, 1991; Mercado *et al.*, 2011; Porté and Loustau, 1998), plant respiration (O'Grady *et al.*, 2010), plant growth and carbon allocation between aboveground and below ground parts (Wang *et al.*, 2012).

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At the canopy level, the *LMA* and nutrient content are prone to substantial temporal variations. Short-term and long-term changes in the leaf nutrition may be caused by various drivers such as leaf ontogeny, herbivory, plant ageing, fertilization, fires, atmospheric depositions, altered precipitation patterns, or nutrient exports by harvests or grazing. Therefore, the comparative assessment of spatial and temporal changes in greenhouse gas, energy and water fluxes between terrestrial ecosystems and the atmosphere, which is the ICOS Ecosystem network's main objective, must account for the spatial and temporal variations in nutritional status of the plant species composing the canopy.

Foliar nutrient analysis is a good proxy widely used as a general diagnostic tool to assess the nutritional status of plant canopies, *e.g.* forest stands (Bauer *et al.*, 1997; Linder, 1995; Rosengren-Brinck and Nihlgard, 1995). Not only the absolute values of mass fraction of each element are requested but the proportions of elements relative to nitrogen are at least as important. Standardised leaves and needles sampling and analyses has been suggested for various purposes (*e.g*. monitor of atmospheric pollution, Eriksson *et al.*, 1989).

The *LMA* is a leaf functional trait commonly used for assessing the ecological status of the foliage of plant species (Cornelissen *et al.,* 2003; Garnier and Narvas, 2011; Poorter *et al.*, 2009; Perez-Harguindeguy *et al.*, 2013). It varies mainly in response to the light regime of the leaf, shaded leaves having a smaller *LMA* than sunlit leaves. The *LMA* is also essential for converting element mass fraction (in kg element × kg dry mass^{-1}) into an area-based content (in kg element × m^{-2} leaf area).

Across the ICOS Ecosystem network, monitoring the foliar nutrient status of the vegetation within the footprint of eddy covariance measurements across sites is critically important. Here, we present standardised guidelines for the measurement of these variables at ICOS Ecosystem sites. The guidelines and definitions provided in this protocol apply to most terrestrial ecosystems. However, there are exceptions such as desert ecosystems where plant leaves are not the main organ through which gaseous and radiative exchanges between vegetation and atmosphere occur, but stems (cacti, mosses) or thallus (lichens) and where specific protocols should be adapted.

Description of the variables

The *LMA* (the term 'specific leaf area', abbreviated SLA, is commonly used and represents the inverse of *LMA*) is the ratio of leaf dry matter mass to its projected area. Dry matter mass is the leaf mass dried at a given temperature until a constant weight is reached. Leaf projected area is taken here as half the total area of the leaf or needle. It is equal to the 'one sided' leaf area for broadleaves. We are aware this simplification does not fully agree with more detailed recommendations given *e.g.* for coniferous needles (Flower-Ellis and Olsson, 1993) for Scots pine and Stenberg et al. (1999) for Norway spruce). This definition is a practical simplification but has also a rationale explained by Chen and Black (1992). These authors have derived mathematically or numerically the projection coefficients of several objects including spheres, cylinders, hemi-circular cylinders, and triangular and square bars for a range of ellipsoidal angular distributions. They showed that the projection coefficient based on half the total intercepting area is close to a constant of 0.5 when the inclination angle of the objects is randomly (spherically) distributed.

LMA is expressed in kg- or g- dry matter × m^{-2} (fresh) leaf area. Typical values range from 50-100 to 150-200 g dry matter mass per m^2 leaf area for broadleaved and coniferous trees respectively (Annex 1, Poorter *et al.*, 2009). The spatial standard deviation of *LMA* within a single species plot can be up to 50%.

The foliar mass fraction (FMF_X) (strictly speaking, the term 'concentration' should point only to the ratio of mass per volume and should not be used here, for clarity. It is however commonly used instead of mass fraction in literature. The word 'content' has no strict definition) where subscript *X* represents any element is the ratio of the element mass to the total dry matter mass of the leaf or needle and is expressed in kg element *X* × kg^1 dry matter. The spatial standard deviation of element mass fraction varies among macro-elements and may be up to 50%. Elements considered in respect with ICOS are carbon, nitrogen, phosphorus, potassium, magnesium and calcium and few microelements iron, copper, zinc and manganese. The values of the foliar mass fraction of selected elements are provided in Annex 2 (extracted from the TRY data basis, November 2015, https://www.try-db.org/TryWeb/Home.php).

Botanically, 'leaf' includes both the petiole and limb in broadleaved species. As for 'needle' this is the entire needle outside the basal scales in coniferous species. For sake of simplicity, the petiole of leaves is excluded from the 'leaf' accounted for in *LMA* and FMF_X determinations.

METHODOLOGY

Sample composition

Two distinct sample categories must be collected together: samples used for the leaf mass to area ratio determination (*LMA*) and samples for the nutrients analysis (NA). Both the *LMA* and NA sample categories are composed of 30 units that include fully expanded, undamaged leaves or needles (Table 1). Each *LMA* unit is composed of one to ten fully expanded leaves/needles or punched leaf disk while each NA unit must include between 10 to 100 fully expanded leaves or about 500 to 1 000 needles in order to reach a total fresh weight between 10 and 30 g per unit for needles 20 and 30 g per unit for leaves. The *LMA* and NA samples must be collected together and paired: for each NA unit collected from a mother plant (element) or a quadrat also one *LMA* unit must be collected from that same mother plant element or quadrat.

Table 1. Composition and size of the sample to be collected for leaf mass to area ratio (*LMA*) and nutrient (NA) determinations

Sample type	Total number (units)	Composition of each unit	Weight of unit fresh mass (g)
LMA sample	30	Entire leaves or needles petiole excluded, Leaf parts, *e.g.* disks, representative of the whole limb or needle	Not applicable
NA sample	30	Entire leaves or needles petiole excluded	10

The foliage elements to be collected must be in the sunlit fraction of the canopy such as south facing branches of the upper third of the tree crowns. If the majority of the foliage area is damaged by fungi, bacteria, insects, nematodes or viruses, a representative sample of the foliage must be collected including damaged leaves.

The species composition of the sample must be representative of the species composition of the ecosystem Green Area Index (GAI) that is half of the total photosynthetically active surface area of standing vegetation, expressed per unit of ground area. For a canopy composed of species A, B and C whose contribution to GAI are 50, 30 and 20% respectively, 15, 9 and 6 units must be collected from trees belonging to species A, B and C, respectively. The basal area (cross sectional area of a tree stem at 1.3 m can be used as a substitute for GAI.

Once defined, the sample composition (number of leaves per unit, number of species sampled *etc*.) should be kept unchanged from year to year unless changes in the canopy composition or structure happen (*e.g.* in croplands with rotation). Changes in the scheme must be always discussed with the ICOS Ecosystem Thematic Centre (ETC).

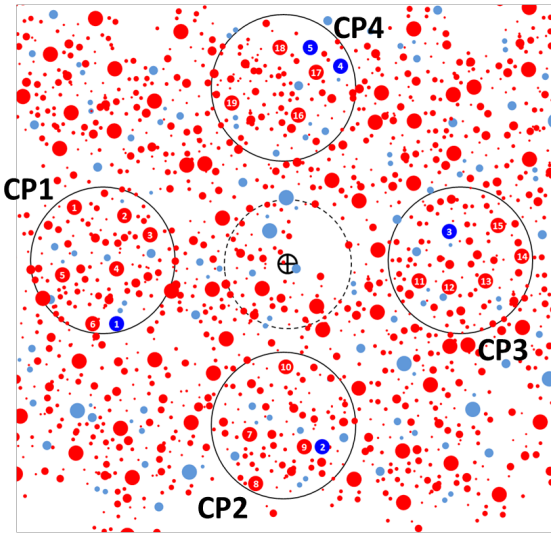

Fig. 1. Example of a class 1 forest station composed of two tree species, red and blue, stems being pictured as circles. The four CPs are reported with a solid black circle, the EC tower is pictured as a cross. In this example, the blue species accounts for one fifth of the total GAI (or basal area). Dominant trees inside the CPs are numbered for each species from 1 to 5 (blue) or 1 to 19 (red). This figure is used as example for the explanation of the two sampling schemes.

Spatial sampling scheme

Forest

The sampling scheme must be defined based on the canopy specific composition, tree inventory and green area index measurements that are assessed during the site characterization. The basal area (horizontal cross sectional area of a tree stem at 1.3 m) can be used as a substitute for GAI. The *LMA* and NA samples must be collected from a minimum of 12 and maximum 30 dominant or co-dominant healthy trees located within the continuous measurement plots (CPs), with a similar number of trees sampled for each CP. In a forest stand, the dominant trees are defined as the 100 thicker trees per ha. For a plot covering *n* hectares, among a list of the diameter of tree stems at 1.3 m height (DBH) ranked by decreasing order, the dominant trees are defined as rank from #(1) to #(100 × *n*). Co-dominant has no strict definition and refers to individual trees having a dominant social status although not strictly speaking dominant. In ecosystems with less than 100 trees per ha, each tree is considered dominant.

The foliage of few broadleaf species, *e.g. Quercus ilex,* and most coniferous species, apart from *Larix*, include several cohorts. However, only the fully expanded needles or leaf of the current year must be sampled.

Ideally, the trees sampled should be kept unchanged from year to year (fixed scheme). However, if repeated collections of foliage samples are expected to damage the trees on the long term, a roving sampling scheme might be used. The two schemes are described below for an example ICOS class 1 forest station in Fig. 1. In both cases leafy twigs are collected in the upper third of the crown from south facing branches. In this example, the mean fresh weight of a single leaf has been fixed to 0.25 g so that the total number of leaves to be collected for NA has been set to 100.

Fixed sampling scheme

For collecting leaves, the trees sampled must be equipped with pulley and ropes and climbed each year (preferred in case of high trees). Each year, the same trees are sampled for NA and *LMA*. Depending on the number of trees sampled up to three units are collected per tree in order to arrive to the total of 30 units requested.

Example based on Fig. 1 the same 15 trees are sampled for NA and *LMA*. For NA, one hundred leaves are collected twice on the same tree. *LMA* and NA samples are collected according to the timetable reported in Table 2. For *LMA*, two samples units are collected in each tree.

Table 2. Fixed sampling scheme proposed. Numbers are the number of units to be collected per tree

Year	Red species																			Blue species				
	Continuous measurement plot (CP)																							
	1						2				3					4				1	2	3	4	
	Tree #																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	1	2	3	4	5
1	2		2	2			2	2		2	2		2	2		2		2	2	2		2		2
2	2		2	2			2	2		2	2		2	2		2		2	2	2		2		2
3	2		2	2			2	2		2	2		2	2		2		2	2	2		2		2
4	2		2	2			2	2		2	2		2	2		2		2	2	2		2		2
5	2		2	2			2	2		2	2		2	2		2		2	2	2		2		2
6	2		2	2			2	2		2	2		2	2		2		2	2	2		2		2

'Blue' and 'Red' refer to the tree species shown in Fig. 1. CP 1 to 4 are the continuous sampling plots used in the ICOS sampling protocol (example in Fig. 1).

Table 3. Roving sampling scheme proposed. Numbers are the number of units to be collected by tree. The numbers underlined are trees being substituted next year

Year	Red species																			Blue species				
	Continuous measurement plot (CP)																							
	1						2				3					4				1	2	3	4	
	Tree #																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	1	2	3	4	5
1	3		2	2			3	2				2		3	2		3	2			3		3	
2	2		3	2				3	2			3	2		2			3	2		3		3	
3	3		2		2			2	3		2	3	3					2	3		2	3		
4	2	3			3			3	2		3		2			2		3	2	3		2		
5		2			2	3			3	2	3		2			3	2		3	2		3		
6		3			3	2	2			3	2			3	2	2	3			3		2		

Explanation as in Table 2.

Roving sampling scheme

In case of trees that can suffer due to the repeated sampling a roving scheme can be applied. This is particularly indicated in case of relatively small trees where leaves can be collected directly by standing on the ground using a pole. To smooth the temporal shift in the sample composition while allowing trees to recover between successive sample collections, one quarter of the trees sampled is renewed every year. In case of remarkable damages due to sampling, the sampling turn-over could be increased up to half.

Example based on Fig. 1: 19 (red) and five (blue) trees are sampled for *LMA* and NA for a total of 12 trees per year. One quarter of trees are renewed every year. With a total of 12 trees sampled, from some of them three sample units must be collected in order to arrive to the 30 units requested. *LMA* and NA samples are collected according to the timetable reported in Table 3.

Grasslands

The *LMA* and NA samples are collected from the SP-II-order locations (second order SP points refer to Spatial Sampling Instruction document). The samples must be taken from 30 SP-II-order points distributed around the 20 SP-I locations, selecting two SP-II sample points on 10 SP-I locations and only one SP-II point on the other 10 SP-I locations. The selected SP-II sample points must include the points chosen for direct aboveground biomass (AGB) measurements by destructive sampling (Instruction on ancillary vegetation measurements in grassland, Fig. 2 as example). The *LMA* and NA samples must be taken at places outside the quadrat used for AGB measurement. The leaves or tillers for *LMA* and NA samples can be picked up randomly in a radius of two meters around each sampling point.

Depending on the specific or plant functional type (PFT) composition of the vegetation, the sample must be split eventually among subsamples corresponding to either main species or PFT (leguminous species, grasses, and non-leguminous forbs). Given the sample size that is fixed to n=30, the sample can be distributed among two to five subsamples. In case the subsamples correspond to PFTs, sampling is distributed between the PFTs in proportion

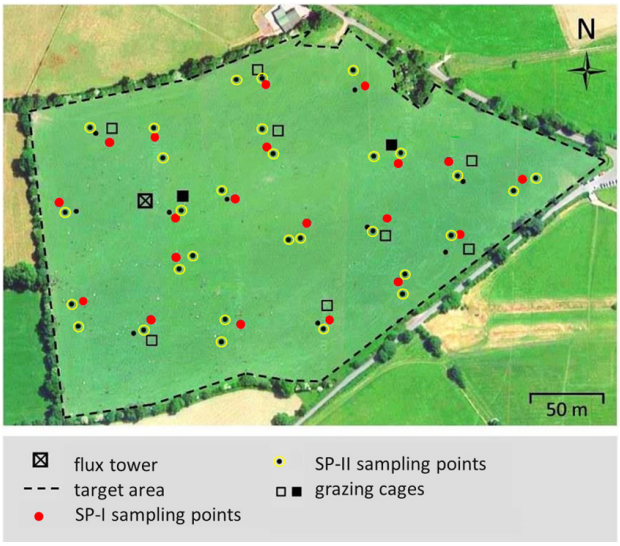

Fig. 2. Spatial sampling scheme for *LMA* and NA at a class 1 grassland station. Thirty measurement points are selected (circled in yellow): one or two second-order Sparse Measurement points (SP-II-order points, black dots) around each of the 20 SP-I-order points (red dots).

with their respective contributions to the total GAI, which is determined in ICOS as part of the ancillary vegetation measurement in grasslands.

Croplands

The *LMA* and NA samples for the 30 units must be collected around the CPs. The leaves or tillers can be picked up randomly. The spatial distribution of samples can be changed according to the species cultivated but must be kept unchanged for the same crop and species throughout the ICOS duration.

Mires

The sampling of vegetation in mire stations is constrained by access facilities. The mire vegetation can be divided into plant community types (between one and maximum four). The CPs are distributed among the community types. Each Principal Investigator of an ICOS Ecosystem station (PI) must therefore adapt a sampling scheme to the site characteristics following the guidelines below:

– first partition the 30 units among the different community types according to their contribution to the LAI (leaf area index) of the target area;
– within each community type distribute the units over selected dominant species, if possible, with a minimum number of three units per species. For the sake of practicality, choose preferentially species that can be sampled at the same dates;
– the samples can be located in the area around the CPs;
– the sampling scheme must be conserved throughout the duration of the observations within ICOS unless drastic changes in vegetation composition happen.

Temporal sampling

NA samples must be collected between one and up to three times per year in ICOS ecosystem class 1 stations and once per year in class 2 stations. *LMA* is measured once a year in both class 1 and 2 stations. The overall sampling procedure must follow the general instructions reported here below. The temporal sampling schemes of *LMA* and NA once adopted must be kept unchanged for the ICOS entire duration.

***LMA* sampling time**

– The *LMA* sampling coincides with one of the three (class 1) or the single (class 2) samplings date for NA. This sampling date has to coincide with a GAI measurement (see Instructions on ancillary vegetation measurements).
– For annual and deciduous species the sampling must be performed immediately after the leaf growth cessation or by the time of maximum GAI, excluding the flowering period (generally May – July).
– For coniferous and other evergreen species the sampling date must be during the dormancy period and just before the eventual hardening period (generally December to February).

NA sampling time

– For all the ecosystems one sampling must coincide with the *LMA* sampling. For class 2 stations this is the only sampling for NA.
– For annual and deciduous species in class 1 stations, the two additional samplings are taken first at the middle of the growing period and last by the end of the growing season before the start of leaf yellowing.
– For evergreen species in class 1 stations, the two additional samplings are one immediately after the cessation of leaf expansion and the other at the end of summer, on current year cohorts.

The temporal sampling depends therefore on the vegetation phenological cycle and may vary by days from year to year. The cultural cycle of crops must also be considered, *i.e.* intermediate winter covers crops. Each Principal Investigator of an ICOS Ecosystem station is requested to define together with the ETC the most appropriate periods for *LMA* and NA sampling and to follow them during the full duration of the ICOS. In Class 1 cropland stations where multiple crops can be cultivated during the same year, it is advisable to sample the different crops as well as winter cover crops. In some cases *e.g.* recently created stations where there is no previous nutrient analysis, a preliminary sampling can be operated for determining the most appropriate sampling period according to the ICOS objectives.

Sample collection in the field

The part of the leaves that must be sampled are the leaf blade and needle, excluding the petiole or the needle included in the basal scales, except when rehydration is needed. The leaves or needles must be clipped from their mother branch or stem with a tool. Contact with hands must be avoided as far as possible. When gloves are used, they should be made of non-powdered vinyl. Other materials such as latex or powdered gloves, contaminating potentially the plant material with elements such as Ca and Mg must be avoided. Samples must never be washed or rinsed. The trees or quadrats from where the samples are collected must be tagged clearly.

Field work preparation

1. Identify the areas where samples will be collected (following the guidelines reported above), possibly before the date of planned sampling in order to register their exact position (coordinates).

2. Plastic bags for *LMA* samples and paper bags for NA samples may be labelled in advance using the code explained at the end of this section. *LMA* plastic bags can be labelled with a permanent marker. NA paper bags must be labelled with a pencil or indelible ink. In addition, a paper tag written with pencil must also be placed inside both the *LMA* and NA bags for double check.

3. Ice packs, cooled from the day before at -30°C, must be placed at the bottom of cooler with a layer of papers above to insulate them with respect to the leaves samples.

Leaves collection

1. Be on site early in the morning for collecting *LMA* samples before leaves start to dehydrate (at dawn ± 2 h).

2. Collect the plant elements from which leaf samples will be taken either manually (by clipping at ground level for crop and grassland) or by shooting or climbing at tree level. Generally, samples of leaves for *LMA* and NA will be picked up then from the same twigs.

3. From each plant element collected, pick up first the leaf/leaves for *LMA* analysis, wrap it in humid paper, place it in the proper pre-labelled *LMA* plastic bag, close the bag hermetically having chased the air from inside and place it in the cooler.

4. In forests, collect leaves for NA determination from the same branch or twigs until a fresh weight of 20-30 g is reached, place them in the proper pre-labelled paper bag, close it with 3-4 staples and store them at a temperature between 5 and 25°C for minimizing freezing risk and volatile compounds losses.

5. For crop and grasslands, leaves for *LMA* and NA should be selected and cut in the laboratory after full hydration.

Leaf area and leaf weight determination

LMA has to be determined entirely by the station staff. Two variables must be measured: the leaf half-area at full turgescence and leaf dry weight. Both of them should be based on entire leaves, needles or tillers, petiole excluded.

Leaf rehydration

Rehydrating leaf samples to full turgescence may be omitted for leaves being not much prone to shrinking (*e.g.* most coniferous and evergreen species or succulent species) and collected just after dawn. For other species, foliar samples must be transported to the laboratory in a cold container at 4°C (not in contact with freezing packs) and rehydrated subsequently as follows:

– cut leaves or tillers/individuals per group/species, place them in deionized water and cut 1-2 mm of the petiole/needle/tiller base under deionized water. Then, without exposing them to air, put the base of the leaf/tiller/needle in a water filled recipient overnight at 4°C;

– the next day, when leaves are fully expanded, separate the last mature leaf from each tiller/individual (in case tillers/individuals were sampled), recut the petiole at the base of the leaf blade and perform the area measurement.

Leaf area measurement

For the measurement of the leaf area needed to calculate the *LMA* three different methods can be used:

Method 1. For leaves with a regular shape, punching leaf disks of known area might be a fast and accurate method. The entire leaf area must be sampled punching the leaf placed above a wooden (hard wood) or plastic board with a set of punchers and a small hammer. Several leaves superposed can be punched together. Put the leaf disks punched from a single unit in a unique paper bag and put

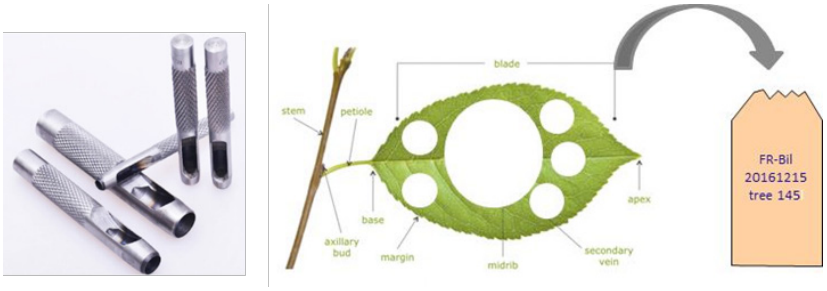

Fig. 3. Calibrated leather puncher (left) and leaf punched for *LMA* determination. Two punchers are used in this example, with area a_1 (small) and a_2 (large), respectively.

the label and the paper tag inside. For the example illustrated in Fig. 3, the total half (one sided) area of disks, A is calculated as follows:

$$A = 5\ a_1 + 1\ a_2, \quad (1)$$

where: a_1 and a_2 are the cross sectional area of punchers 1 and 2, respectively.

Method 2. It might be appropriate to use a planimeter (*e.g.* a LI-3100C Area Meter) or portable flatbed scanner to scan leaves in the field and calculate the area of the scanned structures with image analysis software (after proper calibration). Place the entire leaf on the scanner screen or planimeter bed and scan or measure directly its area at 1% accuracy. In case of non-flat leaf shapes, the scanned area is not the same as the half area. Annex 3 lists some conversion formulae to transform the planimeter area to half area for six non flat geometric leaf shapes.

Method 3. Depending on the needle or leaf shape and size, other techniques may be used for determining the half area of fresh material (electronic caliper, high resolution digital photographs including a proper scale, microscope). Coniferous needles are usually not perfect geometric shapes; in such case, their half area A, may be estimated by piecewise integration from the base to the tip using piece length (l_p) and cross sectional perimeter (s_p) as follows:

$$A = \frac{1}{2}\sum_{p=1}^{n}\left(\frac{l_{p-1}+l_p}{2}\right)s_p. \quad (2)$$

To do this, the perimeter of needle pieces must be determined from thin sections operated by microtome from fresh needles. Thin sections perimeter can be determined then on digital photographs taken with a microscope or binocular.

Whichever method is used to measure the leaf area, at the end of the procedure a leaf area value for each of the 30 units must be calculated summing up all the areas of the leaves/leaf disks punched coming from the same unit. The leaf area measurement method selected must be used and traceable throughout the ICOS duration.

Dry weight determination

Immediately after the leaf area measurement, dry the 30 units of known area at a constant weight in a ventilated oven at 65°C. For the sake of practicality, the duration requested to reach a constant weight can be determined prior to the experiment, typically 24 or 48 h. The temperature of the oven should be controlled by a calibrated sensor and kept between 64 and 66°C. After drying, the samples must be taken from the oven directly into a desiccator filled with silica gel for cooling down to room temperature. Each sample is then weighed to the nearest 0.1 mg. Plant material once dried may rehydrate quickly from air humidity making it necessary to place also a plate filled of fresh dry silica gel near the scale. Dry weight uncertainty is calculated using the uncertainty of the scales used to perform the weighing. The uncertainty of the scales is given by the accredited society that verifies them once a year.

NA samples packaging

The NA samples must be transported to the laboratory and dried at room temperature at approximately 15 to 20°C not more than four hours after the last leaf separation. Each sample must be withdrawn from its paper bag and placed flat in an uncovered aluminum plate on a bench for 48-72 h in a clean open place at room temperature. In case of longer transportation duration, drying should be achieved on site. Each dry sample unit must then be put in its paper bag duly labelled. Great care must be taken to mark each unit clearly before sending it to the Central Plant Analysis Laboratory (CPAL) of the ETC. These identifications must be kept doubled, first on the outer side of the envelope by pencil or indelible ink and second with a label inserted inside each envelope. All units of the same sample should be packed together and the samples should be sent immediately after drying to the CPAL using fast mail (24-48 h delivery).

Maintenance of tools

The calibration of scanner, image analysis software and planimeter must be carried out with calibrated pieces of plastic or steel shapes *e.g.* provided by planimeter manufacturer. Calibration must be carried out before each set of area measurements. The calibration and uncertainty of the scales is given by the accredited companies that should verify them once a year. The thermometer used for oven temperature control must be calibrated by an independent company habilitated for certification by national or international bodies to the nearest 0.5°C.

The area of a puncher mouth can be checked using sheets of paper of know mass and area that can be easily punched to dozen of disks and weighed. The procedure to follow is explained here below:

1. Leave 10 sheets of paper A4 size on a bench overnight, check their size to the nearest mm (210 × 297 mm) and weigh them to the nearest 0.1 mg with the same instrument used for the leaves sample.

2. Calculate their LMA_p as:

$$LMA_p = \frac{W_p}{A_p}, \quad (3)$$

where: the leaf mass area of the paper (LMA_p), A_p is the area given by 210 × 297 × 10 mm², and W_p the weight of 10 paper sheets.

3. Stack them and punch them with the punchers used for extruding 100 discs. Take care to collect the discs from the different parts of the paper sheets

4. The area of the puncher (a) is then calculated as:

$$a = \frac{W_{100}}{LMA_p\ 100}, \quad (4)$$

where: a is the area of the puncher, W_{100} is the total weight of the 100 discs.

Processing

Leaves analysis (C, N, P, K, Ca, Mg, Mn, Fe, Cu, Zn)

Once received in the CPAL, the sample pre-treatment will include drying and grinding. The grinders to be used within ICOS have been tested to prove that no contamination occurs during grinding. Instruments for C, N analysis are available from different manufacturer (Perkin Elmer, Thermo Fisher, Elementar, *etc*.). To analyse C, N, P, K Ca and Mg elements, the most used multi-elemental instrument is ICP-OES (Inductively Coupled Plasma – Optical Emission Spectrometer). ICP-OES are provided by many manufacturer (Agilent, Perkin Elmer, Thermo Fisher). The ETC central laboratory is accredited for analysis of C, N, P, K Ca and Mg with those instruments and regularly participates in inter-laboratory comparison tests for leave analyses (Fürst, 2015). Alternative techniques such as Xray fluorescence could be used. If so, in order to ensure comparability of results along time, the alternative technique must demonstrate its accuracy and precision and its consistency with the previous technique before it can be considered acceptable for ICOS. The measurements performed to each of the 30 units submitted are reported in Table 4. They include the macroelements listed above and some relevant microelements as well.

Table 4. Variables measured on the samples sent for nutrient analysis

Variable	Units
Dry weight	g_{DM}
Dry weight at 65°C	g_{DM}
C content	g C kg_{DM}^{-1}
N content	g N kg_{DM}^{-1}
P content	g P kg_{DM}^{-1}
K content	g K kg_{DM}^{-1}
Ca content	g Ca kg_{DM}^{-1}
Mg content	g Mg kg_{DM}^{-1}
Mn content	g Mn kg_{DM}^{-1}
Cu content	g Cu kg_{DM}^{-1}
Fe content	g Fe kg_{DM}^{-1}
Zn content	g Zn kg_{DM}^{-1}

DM = dry matter.

Time consistency

For each type of measurement, technical improvements can occur during the 20 year time frame of ICOS. These improvements may concern for instance a decrease of quantification limits or an increase in stability and may therefore improve analytical performances. In order to ensure comparability of results along time, performance of a new apparatus will be compared with the old one (using for instance norm XP V03-111:1995) and validated using international standards of leaves and needles. Specifications are given in several norms (BS EN ISO 16634-1:2008, DD CEN ISO/TS 16634-2:2009 and BS EN ISO 11885:2009).

Uncertainty

The uncertainty due to analytical method used is calculated using norm NF ISO 11352 (2013). As this norm is intended to water analysis, it is not exactly suitable for plants analysis. As a consequence the precision is calculated as the standard deviation of a series of a minimum of 20 measurements repeated along a time lap of one year on standard samples representing a wide set of different plant matrices (flour, leaves, needles) corresponding to the matrices studied in ICOS. The accuracy of the nutrient analysis is also assessed from these repeated measurements by comparing the results of the analysis to the 'true' value of the composition of international standards. The uncertainty (precision and accuracy) is controlled every year. An uncertainty value will be given each year with each couple element/analytical method (including solubilising step and instrumental analysis step). The recent norm T90-220: ISO 11352:2012 (February 2013) is being used since 2015.

Calculation for *LMA* determination

The calculation of *LMA*, mean *LMA* and standard deviation will be done by the ETC as follows. *LMA* is expressed in kg dry matter at 65°C per m^2 fresh leaf area (kg DM $m\ leaf^{-2}$). LMA_i of a single sample unit i (1-30) is given by the ratio of the leaf dry weight (W_i) to its fresh area (A_i):

$$LMA_i = \frac{W_i}{A_i}, \quad (5)$$

The leaf area A_i is one-sided which means half the total area whatever the leaf or needle shape. The mean *LMA* value per site, $\overline{LMA}$, is calculated as the average of the n units:

$$\overline{LMA} = \frac{1}{n}\sum_{i=1}^{n} LMA_i. \quad (6)$$

The spatial variability is given by the standard deviation SD_{LMA}:

$$SD_{LMA} = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(LMA_i - \overline{LMA})^2}, \quad (7)$$

when relevant, for mixed stands, vegetation covers community-weighted mean $\overline{LMA}$ is calculated as well.

RESULTS AND DISCUSSION

The spatial variances in *LMA* and element mass fraction within a plant canopy are substantial whatever land use types: crops (Vieira *et al.*, 2010), grasslands (Bump *et al.*, 2009) or forests (Batos *et al.*, 2014; Duquesnay *et al.*, 2000). The spatial variance of both *LMA* and mass fraction includes several components: within a single plant, between individual plants, among species and according to leaf age and light regime (Meir *et al.*, 2002). Significant

spatial variations occur at plot and landscape level as linked to the spatial distribution of soil chemistry, fertilisation, precipitation, flooding, irradiance, atmospheric deposition or the presence of carcasses of large animals (Batos *et al.*, 2014; Bump *et al.*, 2009; Gonzalez *et al.*, 2010; Meziane and Shipley, 1999; Skidmore *et al.*, 2010; Shipley, 2002). Spatial variability may be more important at the early stages of leaf growth and reduced with leaf age (Orgeas *et al.*, 2003). Duquesnay *et al.* (2000) showed that a sample including 13 individuals was sufficient to detect a 5% change in N or P concentrations between two dates in European beech forests. As little as 5 individuals have been shown to lead to detecting a 10% change in element mass fraction for beech forests. However, such a sample size is only sufficient when both samplings are done on the same part of the crown (upper third) and at the same time of the year where nutrient contents are stable. In grasslands, *LMA* and FMF_X vary spatially as a result of variability in species composition and growth. These often reflect local soil conditions and species spreading patterns (Hutchings *et al.*, 2003; Maestre *et al.*, 2006). In grazed grasslands, this variability also results from selective grazing and trampling of vegetation by herbivores (Bakker *et al.*, 2003; Shiyomi *et al.*, 1998) and from patchy deposition of nutrients in animal excreta (Ledgard *et al.*, 1982; Steele, 1982).

The temporal changes in *LMA* and FMF_X may be substantial. Diel variations are linked mostly to starch accumulation in the leaf during the day. Seasonal variations occur in relation with leaf ontogeny and senescence (Aerts, 1996; Bauer *et al.,* 1997; Le Tacon and Toutain, 1973; Migita *et al.*, 2007; Orgeas *et al.*, 2003) and with environmental stresses. There is a strong seasonal variation in *LMA* as an effect of starch accumulation, which also affects FMF_X (Fig. 3 in Linder, 1995, for conifers). Plant age plays also a role, especially for forest trees (Turner *et al.*, 2009). Inter-annual changes are also currently observed. On the long term, climate change, nitrogen deposition, increased atmospheric concentrations of carbon dioxide (CO_2) or soil acidification may induce changes in foliar nutrient mass ratio and phytochemistry in natural ecosystems (Conroy, 1992; Cotrufo *et al.*, 1998; Duquesnay *et al.*, 2000; Jamieson *et al.*, 2015). For instance, Jonard *et al.* (2009) showed that the foliar content of the main broadleaved European species has changed by up to 15% from 1993 to 2005, at monitoring plots in France and Wallonia, positively for N, and negatively for P and Ca. Jonard *et al.* (2015) found similar trends for the main species based long-term monitoring on permanent plots distributed across Europe.

CONCLUSIONS

1. Considering the spatial variability for leaf mass-to-area ratio and foliar mass fractions and the resource allocated to field sampling and chemical analysis, a detailed assessment of the spatial distribution of leaf mass-to-area ratio and foliar mass fractions within the target area of the eddy covariance measurements is too demanding. Instead, the establishment of a consistent and meaningful time series along 20 years is prioritized.

2. The method of regular foliar sampling and analyses used in ICOS allows to monitor the long-term development of the value and the variability of leaf mass-to-area ratio and foliar mass fractions of the main plant species composing the canopy with an uncertainty of typically about 5%. The gained values are however comparable over time, comparable to values in literature and comparable to foliar analyses of other networks (*e.g*. ICP Forests, Rautio *et al*., 2016). They provide a widely accepted basis for (i) the nutritional status and (ii) a leaf functional traits that is related to the ecological status.

3. The ICOS sampling strategy has three practical implications as far as the sampling scheme is concerned.

– First, in order to overcome vertical heterogeneity created within the canopy by the light regime or agricultural management (crop and grass), we suggest to sample only the upper third of the sunlit canopy foliage for forests and individual plants of the overstorey and the last mature leaf for crop and grasslands, respectively. For the case of irregular canopies such as forest stands with contrasted social status only the highest, dominant, individuals are sampled. Therefore, the method does not fully cover *e.g.* shaded leaves and the nutrient pool in the green tissues on the plot cannot thus be assessed using the leaf mass-to-area ratio and nutrients analysis values obtained.

– Second, the sampling design adopted must be kept unchanged during 20 years as far as possible even in the case of complex multi-species or multi layered canopy. However, the foliage sample composition and timing could be changed in few cases: following a clear-cut or a selective thinning of a forest stand, for crop successions, after regular grasslands harvests *etc*.

– Third, depending on the land use type and canopy composition, only plant species contributing significantly to the biogeochemical cycles of GHG are sampled.

Conflict of interest: The Authors declare no conflict of interest.

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Annex 1.

From Poorter *et al*., 2009.

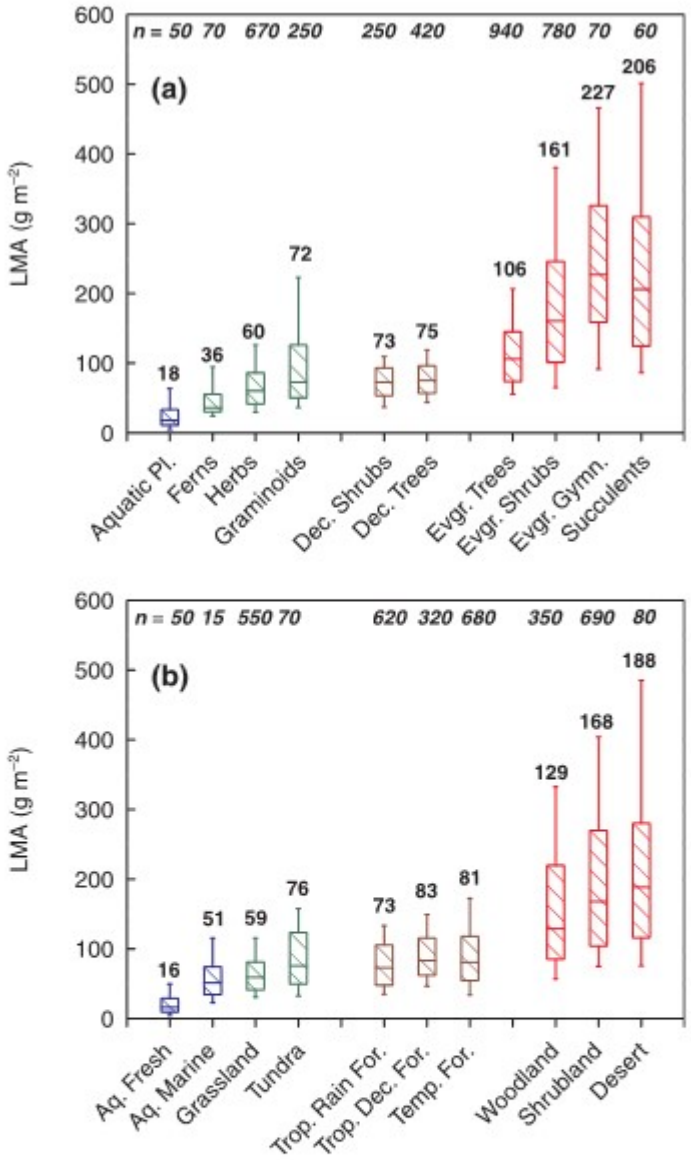
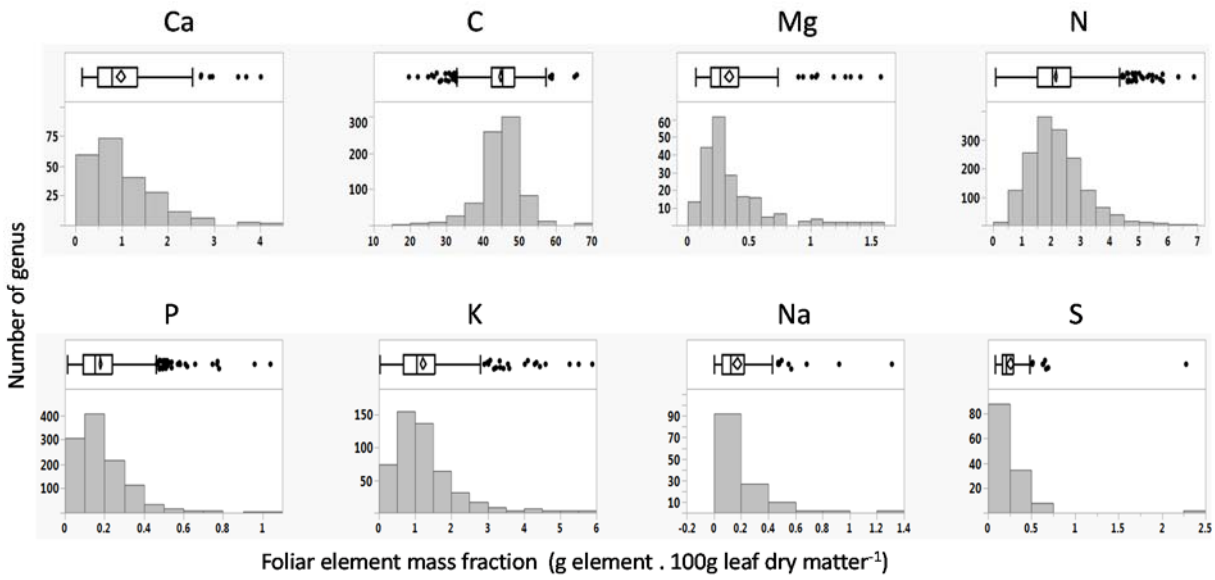


Fig. 1 Distribution of leaf dry mass per unit area (LMA) values in the field, as observed for a wide range of species from (a) different functional groups and (b) different habitats. Box plots characterize this distribution, with the bottom and top part of the box indicating the 25th and 75th percentile, respectively, the two whiskers the 10th and the 90th percentile, respectively, and the horizontal line within the box the median value. The median value is also printed right above the box plots. The total number of species present in each functional group or habitat is indicated at the top of the graphs. More information is given in Appendix A2. *Post-hoc* tests showed that most functional groups were significantly different from at least the groups beyond the adjacent groups in the graphs.

Annex 2.
Values of mass fraction calculated from data extracted from the TRY databasis, November 2015.



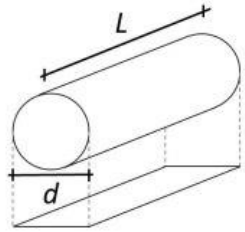
Histograms show the distribution of mean foliar element mass fraction per plant genus for 8 elements as calculated from raw data extracted from the plant trait TRY Databasis (https://www.try-db.org/TryWeb/Home.php). Raw data were first converted in g element × 100g dry mass^{-1} and then averaged by plant genus. Box plots give 95% range, median and mean value (diamond) and show outliers as black dots. The inset shows the mean value in g element × 100g dry foliar mass^{-1} and sample size (number of genus).

Caution: Dry mass may have been determined at different temperatures (60 to 105 °C) and analytical methods may vary among data sources as well. Leaf petiole may or may not be included.

Annex 3.

For non-flat leaves and needles, the area measured with the planimeter or projected on the scanner is not the half area. It has therefore to be converted. In the figure below, you find for six common geometric leaf and stem shapes how to calculate half area (A_L) from scanned area (A_{scan}).

cylinder



basal area is excluded

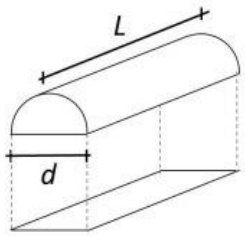
$$A_L = \frac{\pi}{2} dL$$

$$A_{scan} \approx dL$$

$$A_L \approx \frac{\pi}{2} A_{scan}$$

$$\approx 1.57 A_{scan}$$

half cylinder
full



basal area is excluded

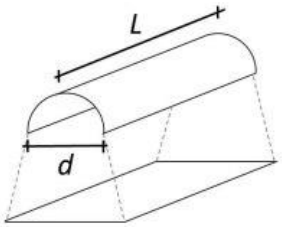
$$A_L = \left(\frac{\pi + 2}{4}\right) dL$$

$$A_{scan} \approx dL$$

$$A_L \approx \left(\frac{\pi + 2}{4}\right) A_{scan}$$

$$\approx 1.29 A_{scan}$$

half cylinder
hollow



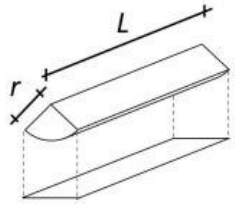
leaf is pushed flat in scanner

$$A_L = \frac{\pi}{2} dL$$

$$A_{scan} \approx \frac{\pi}{2} dL$$

$$A_L \approx A_{scan}$$

quarter cylinder



basal area is excluded
lay on rounded side

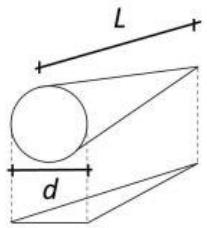
$$A_L = \left(\frac{\pi + 4}{4}\right) rL$$

$$A_{scan} \approx \sqrt{2} rL$$

$$A_L \approx \frac{(\pi + 4)}{4\sqrt{2}} A_{scan}$$

$$\approx 1.26 A_{scan}$$

cone



basal area is excluded
we assume L >> d

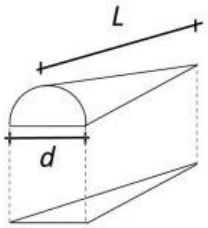
$$A_L = \frac{\pi}{4} dL$$

$$A_{scan} \approx 0.5 dL$$

$$A_L \approx \frac{\pi}{2} A_{scan}$$

$$\approx 1.57 A_{scan}$$

cut cone



basal area is excluded
we assume L >> d

$$A_L = \left(\frac{\pi + 2}{8}\right) dL$$

$$A_{scan} \approx 0.5 dL$$

$$A_L \approx \left(\frac{\pi + 2}{4}\right) A_{scan}$$

$$\approx 1.29 A_{scan}$$