ICOS Ecosystem Thematic Centre

ICOS Ecosystem Instructions

FOLIAR SAMPLING AND LMA

Version: **20210614**

INSTRUCTIONS FOR

FOLIAR SAMPLES COLLECTION AND LEAF MASS TO AREA RATIO DETERMINATION

Version	Release date	Summary of changes
20180415	20180415	Changed the address to ship the samples. Clarified that metadata and correct labelling of samples are mandatory for the analysis.
20210614	20210614	Added specific explanations for mires

The ICOS protocols and the derived Instructions documents can be changed and amended in time, because new methods become available or to improve their clearness. For this reason, it is crucial to keep track of the versions and differences.

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SUMMARY

The nutritional status of the canopy foliage in terms of macronutrients (C, N, P, K, Ca, Mg, Fe, Cu, Mn, Zn) has a strong influence on the carbon cycle and energy balance of terrestrial ecosystems and therefore on the energy, water and greenhouse gas fluxes between terrestrial ecosystems and the atmosphere. The leaf mass to area ratio, LMA, is also a widely used leaf functional trait the spatial and temporal variations of which are essential for interpreting canopy exchanges of energy, and CO_2. Foliage macronutrient contents and LMA will be assessed at ICOS Ecosystem stations from nutrient analyses and mass/area measurements on periodically collected foliar samples. This document describes how the foliar samples must be selected and taken up and how LMA must be measured at ICOS Ecosystem stations. It also describes how to prepare and ship samples to ETC's analytical lab, where the nutrient analyses are carried out.

The document is structured in different sections with the aim to facilitate the use and application:

- Sampling collection and measurements: the general strategy used for the sampling is shortly described. The tools and method used to take the leaves or needles composing the samples together with the collection of metadata requested are presented in order to practically complete the foliage sampling and LMA determination.
- Maintenance: in this section the possible maintenance activities are reported.
- Submission: it is the section related to the data preparation and submission to the ETC, including the foliar samples. It includes the list of parameters and the way to submit them to the database. It is linked to the BADM system.
- Processing: this section describes first how leaf nutrient will be determined by the ETC laboratory and second how the LMA will be calculated.

It is important to remind that the exact application of the protocol at station level must be also discussed with ETC in order to reach agreed solutions for specific cases. If a specific part is relevant only for a specific ecosystem or site Class this is also reported in the document.

This Instruction document is based on the following ICOS Ecosystem protocols:

- Loustau et al: "Protocol for foliar analysis"
- ETC Instruction document "Instructions_ECO_Sampling_Design"
- ETC instruction document "Instructions_ECO_station_description"
- Op de Beeck et al. "Ancillary vegetation measurements in mires"
- Op de Beeck et al. "Ancillary vegetation measurements in grasslands"
- Gielen et al. "Ancillary vegetation measurements in forest"
- Gielen et al. "Ancillary vegetation measurements in croplands"

SAMPLE COLLECTION AND MEASUREMENTS

The sample collection and measurements are done manually and include the following steps:

- Collection of foliar samples for nutrient analysis,
- Collection of foliar samples for LMA determination,
- Sample drying and packaging for shipment to central ETC laboratory for nutrient analysis,
- LMA determination.

Sensors and Material needed

Common material needed for all the ecosystems:

- Safety equipment;
- Labelling system (ribbons, tape, metal tags,…) for tagging sampled plants;
- GPS, decameter, compass;
- Sharpened scissors, cutter, knife or scalpel, clippers, plyers;
- Sharpened puncher of know diameter (when punching method is used);
- 40 paper bags of 0.5-1.0 dm^3 volume (30 + 10 spare);
- 40 PVC or polypropylene waterproof bags of 0.25-0.5 dm^3 volume (30 + 10 spare);
- Icebox with ice packs;
- Indelible permanent markers;
- Paper labels (2 x 5 cm);
- Black pencil;
- Stapler;
- Fastenings;
- Plastic PVC sheet 1.5 x 2m;
- 1 dm^3 volume washed bottle filled with water;
- Planimeter or scanner or any system allowing to determining the area of leaf samples with >1% accuracy;
- Ladder or climbing equipment or shooting equipment (see below shooting and climbing);
- Tree pruner.

Material needed for samples drying:

- Ventilated oven at constant temperature including a calibrated thermometer;
- Desiccator filled with freshly regenerated silica gel.

Material needed for weighing and area determination:

- Electronic weighing scale, with a measurement precision of 0.1 mg;

- Planimeter (LI-3100A, LI-3100C, WinDIAS etc.) or any verified digitizer device or flatbed scanner + image analysis software.

Material needed in forest ecosystems when shooting is used:

- Shotgun, cartridges, ear protection, eye protection, shoulder protection;
- Official habilitation and allowance for shooting in forest;
- Warning signals for safety zone delimitation.

Material needed in forest ecosystems when climbing is used:

- Certified habilitation and training to perform the activity;
- Throwline with bag;
- Throw weight and launcher;
- Access rope;
- Rappel rope;
- Anchor slings;
- Harness;
- Helmet;
- Gloves;
- Rope snaps, double blocking carabiners, footlock prusiks, hip prusiks, eye and eye prusik;
- All the material requested by the certification and training.

Foliar samples: common features

There are two distinct samples categories that must be collected: samples used for the nutrients analysis (NA) and samples for the leaf mass to area ratio determination (LMA). Both the NA and LMA sample categories are composed of 30 units that include fully expanded, undamaged leaves or needles. 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 hence damaged leaves.

Each LMA unit is composed of either fully expanded leaves/needles or punched leaf disks. The number of leaves per unit may vary depending of the leaf size from one to ten. The number of leaf disks per unit must be at least five taken from one or several leaves. Depending on the vegetation type, each NA unit must include 10 to 100 fully expanded leaves in order to reach a total fresh weight between 20 and 30 g per unit.

The NA and LMA samples are distinct but must be collected together and paired: for each NA unit collected from a mother plant (element) or in a measurement quadrat also one LMA unit must be collected from that same mother plant (element) or in that same measurement quadrat.

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 ETC.

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 major elements (Ca, Mg…), must be avoided. Samples must never be washed or rinsed.

The trees, mother plants or measurement quadrats from where the samples are collected must be tagged clearly.

Sampling scheme

Forest

The sampling scheme must be defined by the station team based on the canopy specific composition, tree inventory and Green Area Index (GAI) or basal area measurements that are assessed during the site characterization. The NA and LMA 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. Where appropriate, it has to be split among species according to their contribution to the total stand GAI or basal area.

Dominant trees: these are the 100 thicker trees per ha. Among a tree DBH list ranked by decreasing order, the dominant trees are defined as rank from #1 to #100. 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 they are all considered dominant.

Splitting by species: for a mixed canopy where species A, B and C contributions to GAI are 50, 30 and 20%, 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 1.3m ($= \Pi \times (DBH / 2)^2$) can be used as a substitute for GAI.

Which cohort should be sampled? 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 after discussion with the ETC. The two schemes are described here below using as example the Figure 1. In both cases leafy twigs are collected in the upper third of the crown from South facing branches and, the mean fresh weight of a single leaf/needle has been fixed

to 0.25g for example so that the total number of leaves/needles to be collected for NA has been set to 100 per unit for each tree species.

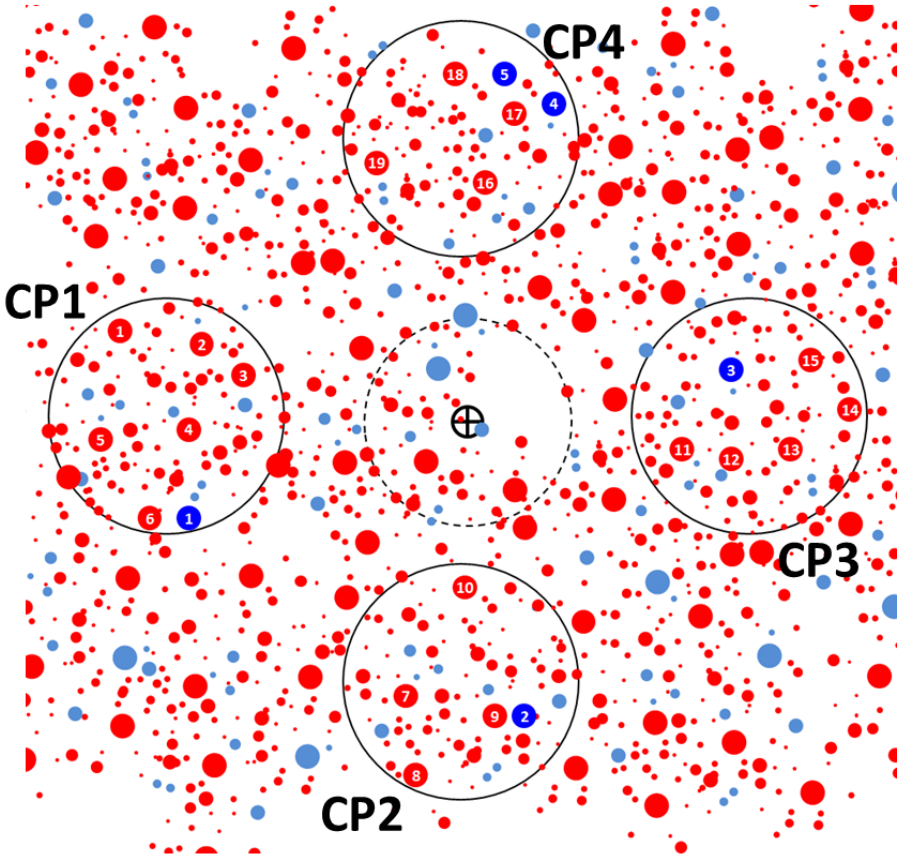


Figure 1. Example of a Class1 forest site composed of two tree species, red and blue, stems being pictured as circles. The four CPs 1-4 are reported with a continuous black line, the EC tower is pictured as a cross. In this example, the blue species accounts for one fifth of the total GAI (or basal area). Dominant trees inside the CPs are numbered for each species from 1 to 5 (blue) or 19 (red). This figure is used as example for the explanation of the two sampling schemes.

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 3 units are collected per tree in order to arrive to the total of 30 units requested.

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

Table 1. Fixed sampling scheme proposed. Numbers are the number of units to be collected per tree.

		Red species																			Blue species				
		CP1						CP2				CP3					CP4				CP1	CP2	CP3	CP4	
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
year	2017	2		2	2			2	2		2	2		2	2		2		2	2	2		2		2
	2018	2		2	2			2	2		2	2		2	2		2		2	2	2		2		2
	2019	2		2	2			2	2		2	2		2	2		2		2	2	2		2		2
	2020	2		2	2			2	2		2	2		2	2		2		2	2	2		2		2
	2021	2		2	2			2	2		2	2		2	2		2		2	2	2		2		2
	2022	2		2	2			2	2		2	2		2	2		2		2	2	2		2		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. Each year, part of the trees sampled is renewed; 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. Would sampling damage be more important, the sampling turn-over could be increased up to half.

Example based on figure 1: 19 (red) and 5 (blue) trees are sampled for NA and LMA 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. NA and LMA samples are collected according to the timetable reported in table 2.

Table 2. Roving Sampling scheme proposed. Numbers are the number of units to be collected by tree. The numbers underlined are trees being substituted next year.

		Red species																			Blue species				
		CP1						CP2				CP3					CP4				CP1	CP2	CP3	CP4	
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
year	2017	3		2	2			3	2				2		3	2		3	2			3		3	
	2018	2		3	2				3	2			3	2		2			3	2		3		3	
	2019	3		2		2			2	3		2	3	3					2	3		2	3		
	2020	2	3			3			3	2		3		2			2		3	2	3		2		
	2021		2			2	3			3	2	3		2			3	2		3	2		3		
	2022		3			3	2	2			3	2			3	2	2	3			3		2		

Grasslands

The NA and LMA 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, thus 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 AGB measurements by destructive sampling (see Instruction on ancillary vegetation measurements in grassland and figure 2 as example). The NA and LMA samples must be

taken at places outside the quadrat used for AGB measurement. The leaves or tillers for NA and LMA samples can be picked up randomly in a radius of 2m around each of the 30 SPII sampling points.

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

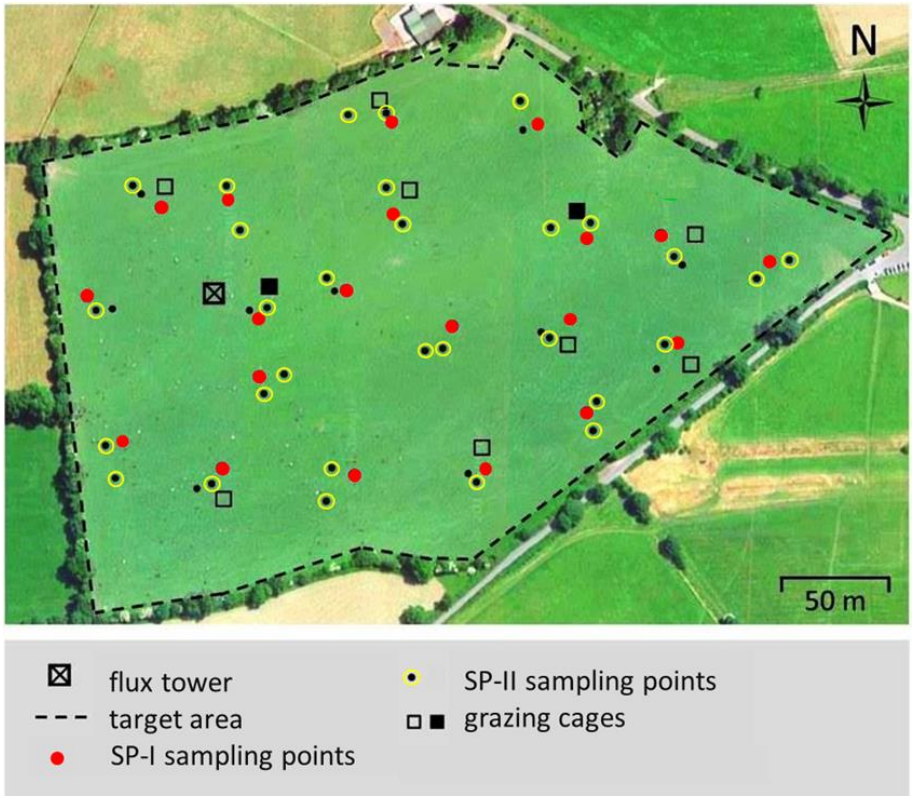


Figure 2. Spatial sampling scheme for NA and LMA in a grassland site. Thirty measurement points are selected (circled in yellow): one or two second-order Sparse Measurement points (SP-II-order points, black dots) around each of the 20 SP-I-order points (red dots).

Croplands

The NA and LMA samples for the 30 units must be collected within the CPs, trying to collect samples uniformly from the different CPs. The leaves or tillers for NA and LMA samples 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 (mosses, graminoids, forbes, shrubs, trees) can be divided into dominant plant species in terms of canopy cover (between 1 and a maximum of 5, see Instructions_ECO_Ancillary_Mires: Mosses Green Area). In addition, the sample might be stratified into one or more plant micro site - hummock, lawn, hollow type when relevant. Each PI must adapt a sampling scheme of NA and LMA accounting for the species % cover in the target area following the guidelines below:

- The sampling scheme must be maintained throughout the ICOS duration unless drastic changes in vegetation composition happen.
- Partition the sample units among one to five plant species having the highest cover percentage of the target area, as it was measured for the site characterization (see Site characterization in mires), accounting for the micro sites distribution when relevant. When the site characterization has not been completed, use any information available, photographs or a visual inspection of the target area to estimate the cover percentage of the main plant species.
- It should be kept in mind that the minimum sample size per species as needed for achieving an accurate estimate of nutrient content is five.

  Example: *The main plant species in the SE-Deg Class2 site are Sphagnum balticum (SB), Sphagnum papillosum (SP), Sphagnum majus (SM) and Sphagnum fuscum (SF), a sampling scheme to be proposed would allocate the 30 sample units as follows.*

  SB: 18; SP: 6; SM:6.

  Including a fourth species, e.g. SF, is debatable because this would diminish too much the number of samples per species.

- The samples can be located at SPII points.
- In sphagnum bogs, since the Sphagnum canopy may take several years to recover after collection, the sampling locations must be renewed in order to avoid resampling the same SPII for the next 2 years. To do this, relocate the samples within 3m from the previous year location.

The site-specific sampling scheme must be agreed with the ETC. Add all necessary information in support of the sampling scheme proposed and send to the responsible person for the foliar analysis in the ETC (with info@icos-etc.eu in CC).

Temporal sampling

NA samples must be collected between once a year in Class 1 stations and once per year in Class 2 stations. However, class 1 stations can send for analysis up to 90 samples per year. Thus, depending of the site vegetation and growing season length, class-1 station teams can propose to include

additional samplings accounting for site vegetation composition, micro site distribution and species phenology.

LMA is measured once a year in both Class 1 and Class 2 stations except for moss species where it is not compulsory.

The overall sampling procedure must follow the general instructions reported here below. The temporal sampling schemes of NA and LMA 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

Each PI is requested to define together with the ETC the most appropriate periods for NA and LMA sampling and to follow them during the full duration of the ICOS. The exact sampling dates are then decided and discussed yearly with ETC.

The temporal sampling depends on the vegetation phenological cycle and may vary by days from year to year. For examples, in croplands Class-1 stations where multiple crops can be cultivated during the same year, it is advisable to sample the different crops and winter cover crops as well. 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. It is the PI's responsibility to determine whether such a preliminary monitoring is requested, e.g. a periodic monthly sampling during one growing season. Alternatively, the annual sampling calendar can be proposed based upon literature data.

For all the ecosystems one sampling must coincide with the LMA sampling. For Class2 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.

- For Sphagnum bogs, the sampling time must be during the second half of the growing season, typically end of August. LMA sampling in mosses is not compulsory. However, specific protocol for LMA determination or a proxy variable such as the spatial density of *capitula* can be proposed as well by station team.

Samples collection guideline

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. Figure 3 shows examples of the part of leaves to be collected.

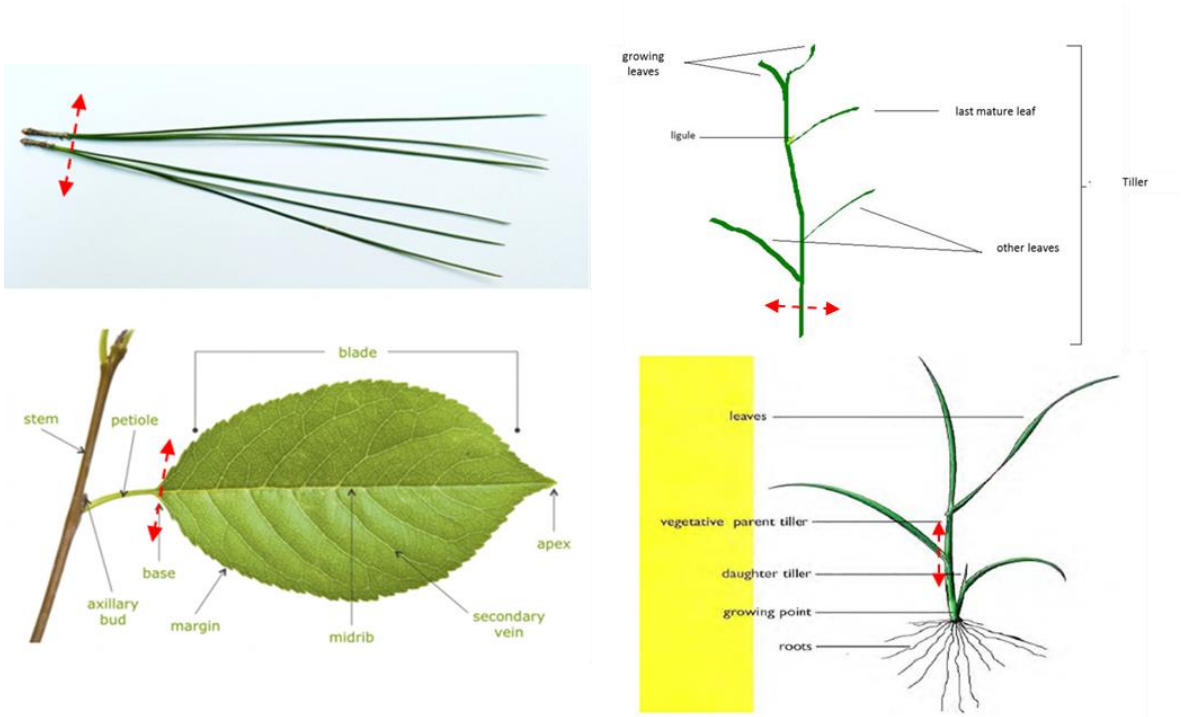


Figure 3 (continue…). Pictures of needle (above, left) and leaves (below, left) and grass (right) showing the parts to be clipped for nutrient analysis and LMA determination.

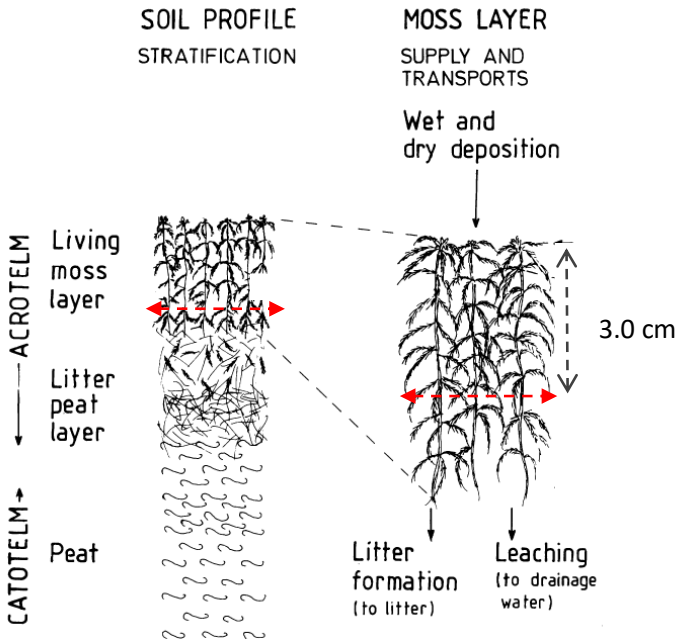
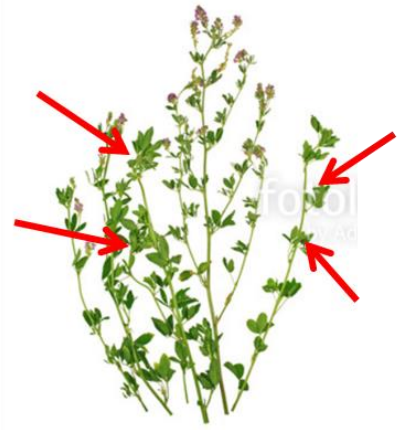


Figure 3 (continue…). Pictures of Sphagnum peat showing the parts to be clipped for nutrient analysis.

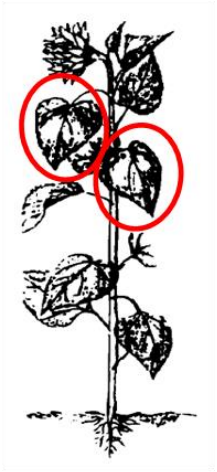
Maize at 4th-leaf stem-elongation phase: fully expanded leaves.



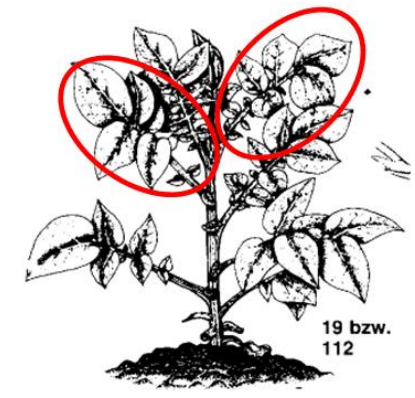
Alfalfa: mature leaves before seed set.

Wheat: mature leaf at the end of the stem elongation phase.

Rapeseed: recently matured leaves before seed set.



Sunflower: recently matured leaves before heading.



Potato: 3rd and 4th leaves before bloom. Compounded leaves include several leaflets

Figure 3 (continued). Plant parts to be clipped for nutrient analysis and LMA determination for some crop species (red arrows and circles). The phenological stage shown here is the end of the vegetative phase. Drawings have been copied from "Growth stages of mono-and dicotyledonous plants", BBCH Monograph, 2. Edition, 2001, Edited by Uwe Meier, Federal Biological Research Centre for Agriculture and Forestry.

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 NA and LMA 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 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 hours).
2) Collect the plant elements from which leaf samples will be taken either manually (by clipping at ground level for cropland, grassland and mire) or by shooting or climbing at tree level. Generally, samples of leaves for NA and LMA 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 croplands and grasslands, leaves for NA and LMA should be selected and cut in the laboratory after full hydration.
6) For the moss species, the upper 3 cm of the moss layer including entire stem, branch and capitulum are collected. The sample must be cleaned from litter, vascular plant roots and other plant material. The amount of material to be collected per unit sample in order to reach a minimum sample weight of 2g dry mass may vary according to species, from approximately 20 (Sph. magellanicum) to >100 individuals.

Leaf area and leaf weight determination (LMA)

LMA has to be determined entirely by the station staff. Two variables must be measured: the leaf half-area at full turgescence and the 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 (Figure 3) 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 the label and the paper tag inside. For the example illustrated in figure 4, the total half (one sided) area of disks, A, is calculated as follows:

$$A = 5 \times a_1 + 1 \times a_2$$

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

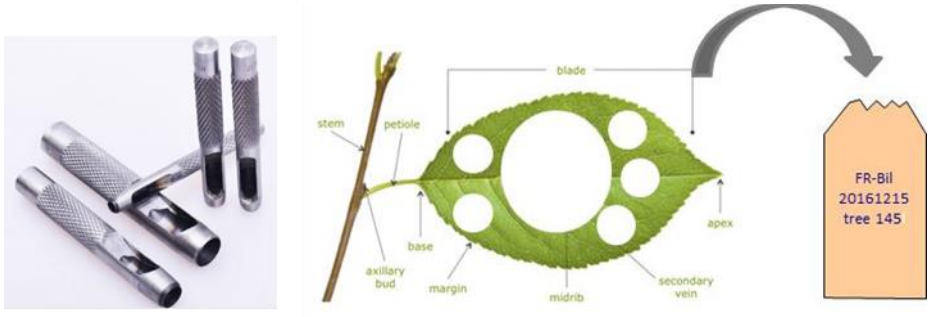


Figure 4. Calibrated leather puncher (left) and leaf punched for LMA determination.

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 1 lists some conversion factors 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 calliper, 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:

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

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 hours. 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 weighted 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 4 hours after the last leaf separation. Each sample must be withdrawn from its paper bag and placed flat in an uncovered aluminum plate (cooking disposals) on a bench for 48-72 hours in a clean open place at room temperature. In case of longer transportation duration, drying should be achieved on site. *Sphagnum* and other moss samples that may be difficult to dry can be placed in a ventilated oven at a maximal temperature of 40°C.

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). 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.

The coding rule to be used must follow the scheme:

CC-###_YYYYMMDD_UU

where *CC-###* is the station code, *YYYYMMDD* is the sampling date and *UU* is the unit number (between 01 and 30). Example: *FR-Bil_20161225_01, FR-Bil_20161225_02, … FR-Bil_20161225_30*

The samples must be then sent to the ETC following the instruction provided in the Submission section of this document, where also the needed metadata are explained.

NOTE: a correct labelling of the samples and a complete metadata are crucial for the quality of the measurements and analysis. For this reason samples with coding not following the standard procedure explained above will not be analysed.

MAITENANCE 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 verifies them once a year.

The thermometer used for oven temperature control must be calibrated by a certified organism 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 x 297 mm) and weigh them to the nearest 0.1mg with the same instrument used for the leaves sample.
2. Calculate their LMA as

$$LMA_p = W_p / A_p$$

   where LMA_p is the "Leaf Mass Area" of the paper, A_p is the area given by $210 \times 297 \times 10$ 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 is then calculated as:

$$a = W_{100} / (LMA_p \times 100)$$

   where a is the area of the puncher, W_{100} is the total weight of the 100 discs and LMA_p is the "Leaf Mass Area" of the paper.

SUBMISSION

NA samples shipping to ETC-CPAL

Samples should be sent immediately after drying to the Central Plant Analysis Laboratory (CPAL) of the ETC using fast mail (24 - 48h delivery). All units of the same sample should be packed together and sent to the following address:

UMR ISPA (Attention: C. Aluome)
ETC Central Laboratory
INRA
71 Avenue Edouard Bourlaux
CS 20032
33882 Villenave d'Ornon cedex
France

Samples must be packed and the carefully labelled as explained in the specific section.

Data and Metadata submission

All the data and information needed to correctly process and document the samples shipped to the ETC must be submitted using the BADM system (FLSM group). Data and metadata cover three main categories: data for the LMA calculation, metadata on the method used for sampling and calculation and metadata to identify and characterize each sample shipped.

All the metadata are mandatory and needed before the samples analysis and for this reason must be submitted as soon as the samples are ready to the shipped. Without metadata the analysis can not be done.

Data for LMA calculation

Data includes all the measured data per each unit and the info on location (referring to CP or SP code, tree ID etc.) and are submitted using the BADM system

Metadata on method used to sample and calculate LMA

Data includes the method and the characteristics of the instruments (oven, weight precision etc.) and are submitted using the BADM system

Metadata on the samples sent

Information needed to link the single sample shipped to the ETC-CPAL (using the sample code) to the location and characteristics where it has been collected.

PROCESSING

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

Once received in the CPAL plant laboratory, the sample pre-treatment will include drying and grinding. Drying cupboard and grinders are normal instruments of laboratory. The grinders to be used for ICOS network 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, ...). The ETC central laboratory is accredited for analysis of C and N with one of these instruments. To analyse major elements, the most used multi-elemental instrument is ICP-OES (Inductively Coupled Plasma – Optical Emission Spectrometer). ICP-OES are provided by many manufacturers (Agilent, Perkin Elmer, Thermo Fisher). The ETC central laboratory is accredited for analysis of P, K Ca, Mg with those instruments. Alternative techniques such as X ray 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 be made consistent with the previous technique before it can be considered acceptable for ICOS. Therefore, it will perform analysis with at least the same quality level so that the consistency of the time series obtained will be guaranteed.

The measurements performed to each of the 30 units submitted are reported in table 3.

Table 3. Variables measured by the ETC on the samples sent. DM = dry matter

Variable	Units
Dry weight	g_{DM}
Dry weight at 65°C	g_{DM}
Dry weight at 105°C	g_{DM}
C content	$gC\ kg_{DM}^{-1}$
N content	$gN\ kg_{DM}^{-1}$
P content	$gP\ kg_{DM}^{-1}$
K content	$gK\ kg_{DM}^{-1}$
Ca content	$gCa\ kg_{DM}^{-1}$
Mg content	$gMg\ kg_{DM}^{-1}$
Cu content	$gCu\ kg_{DM}^{-1}$
Fe content	$gFe\ kg_{DM}^{-1}$
Mn content	$gMn\ kg_{DM}^{-1}$
Zn content	$gZn\ kg_{DM}^{-1}$

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. It may not have negative impact on measurements for ICOS. 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 XP T 90-220:2003. As this norm is dedicated to water analysis, it is not exactly suitable for plants analysis. As a consequence the uncertainty is calculated thanks to reproducibility measurements during a long period of time (at least n=20 during 1 year) and thanks to inter-laboratory comparisons representing a wide set of different plant matrices (flour, leaves, needles) corresponding to the matrices studied in ICOS. The uncertainty is controlled every year. An uncertainty value will be given each year with each couple element/analytical method (including solubilization step and instrumental analysis step). The new norm T90-220: ISO 11352:2012 (February 2013) may be used directly in the future.

Calculation for LMA determination

The calculation of LMA values, 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 at 105°C will be also estimated from the water content difference from 65°C to 105°C measured at the CPAL. 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):

$$LMA_i = W_i / A_i$$

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 is calculated as the average of the *n* units:

$$\overline{LMA} = \frac{1}{n}\sum_{i=1}^{n} LMA_i$$

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

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

Community weighted mean

When relevant, for mixed stands, vegetation covers community-weighted mean LMA (LMA_c) is as well calculated. Typical LMA values range from 50-75 to 175-225 g dry matter mass per leaf area for broadleaves and needles, respectively. The spatial standard deviation of LMA within a single species plot can be up to 50%.

References

Kruk, M., and Podbielska, K.: Potential changes of elemental stoichiometry and vegetation production in an ombrotrophic peatland in the condition of moderate nitrogen deposition, Aquatic Botany, 147, 24-33, https://doi.org/10.1016/j.aquabot.2018.03.004, 2018.

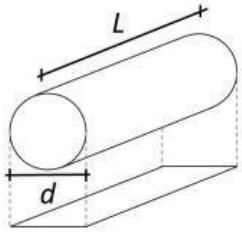
Limpens J., Bohlin E., Nilsson M.B., 2017. Phylogenetic or environmental control on the elemental and organo-chemical composition of Sphagnum mosses ? Plant Soil, DOI 10.1007//s11104-017-3239-4

Malmer, N.: Patterns in the Growth and the Accumulation of Inorganic Constituents in the Sphagnum Cover on Ombrotrophic Bogs in Scandinavia, Oikos, 53, 105-120, 10.2307/3565670, 1988.

ANNEX 1 - Converting projected area to half surface area

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



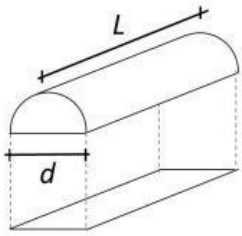
basal area is excluded

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

$$A_{scan} \approx dL$$

$$A_L \approx \frac{\pi}{2} A_{scan} \approx 1.57 A_{scan}$$

half cylinder
full



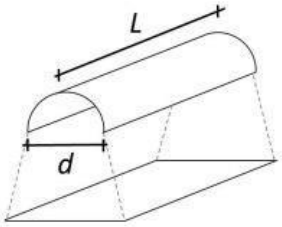
basal area is excluded

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

$$A_{scan} \approx dL$$

$$A_L \approx \left(\frac{\pi + 2}{4}\right) A_{scan} \approx 1.29 A_{scan}$$

half cylinder
hollow



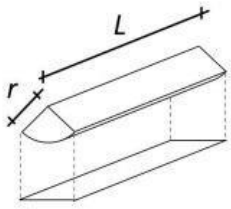
leaf is pushed flat in scanner

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

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

$$A_L \approx A_{scan}$$

quarter cylinder



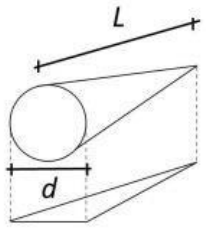
basal area is excluded
lay on rounded side

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

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

$$A_L \approx \frac{(\pi + 4)}{4\sqrt{2}} A_{scan} \approx 1.26 A_{scan}$$

cone



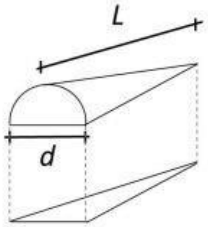
basal area is excluded
we assume L >> d

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

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

$$A_L \approx \frac{\pi}{2} A_{scan} \approx 1.57 A_{scan}$$

cut cone



basal area is excluded
we assume L >> d

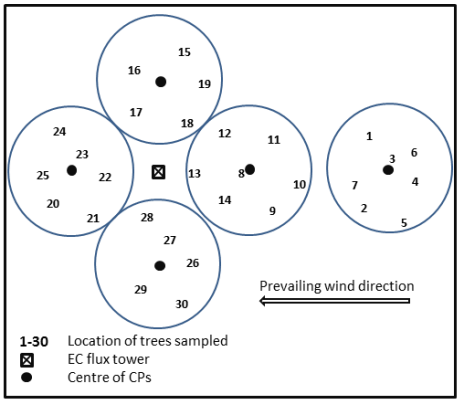
$$A_L = \left(\frac{\pi + 2}{8}\right) dL$$

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

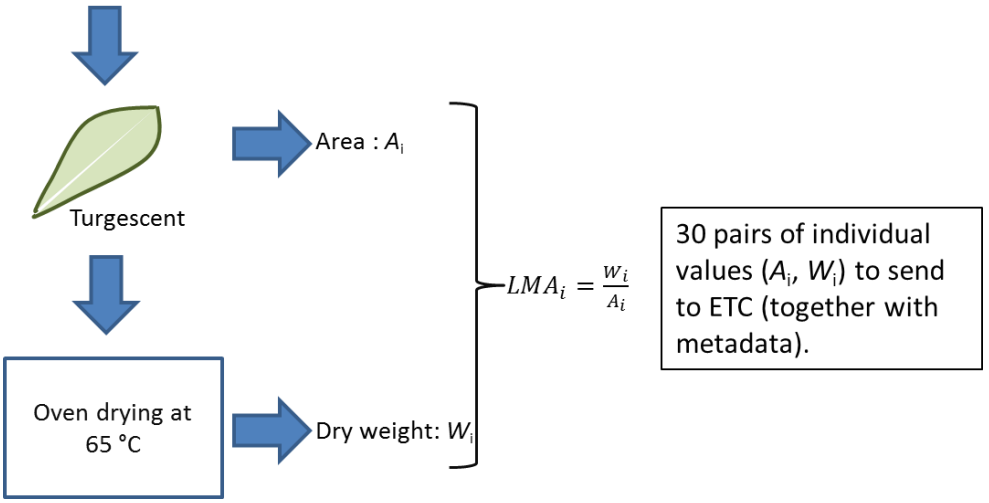
$$A_L \approx \left(\frac{\pi + 2}{4}\right) A_{scan} \approx 1.29 A_{scan}$$

ANNEX 2 – Summary of the protocol for LMA

The picture below summarises, as example, the protocol to be applied for a monospecific canopy with 5 CPs.

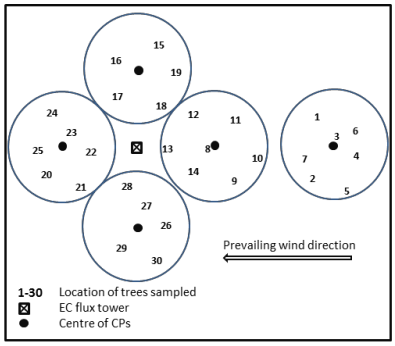


30 units collected from 2 (class 2) to 5 CPs (class 1).
For each unit *i*, (an entire leaf here):



ANNEX 3 – Summary of the protocol for NA sampling

The picture below summarises, as example, the protocol to be applied for a monospecific forest canopy with 5 CPs.



Sample composed of 30 units collected from 2 (class 2) to 5 CPs (class 1).
Transportation to the lab, air dried at room temperature, put in 30 separate bags.

FR-BIL 20170101 -01 × 30 + data sheet

Variable	Description	Units	dataValue
SITE_ID	Six character site identifier. MANDATORY if site is already registered	CC-Xxx	*FR-BIL*
SITE_NAME	Site name	free text	*Salles*
SUBMISSION_CONTACT_NAME	Name of person who submitted this template	free text	*Loustau*
SUBMISSION_CONTACT_EMAIL	E-mail address of the person who submitted this template	free text	*Denis.loustau@bordeaux.inra.fr*
SUBMISSION_DATE	Date this submission was last edited/modified.	YYYYMMDD	*20140115*
BIOMASS_ORGAN	Organ for which the biomass concentrations are reported	LST(ORGAN_ALL)	Foliage
BIOMASS_PHEN	Phenology of the organ	LST(ORGAN_PHEN)	Green
BIOMASS_SPP	Biomass C and N concentration species or plant functional type	Scientific name or PFT	Pinus pinaster
BIOMASS_APPROACH	Biomass C and N concentration measurement approach	free text	ICOS protocol
BIOMASS_COMMENT	Biomass C and N concentration comments	free text	*This data is for sample SALLES-20140102-number 1 to 30*

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