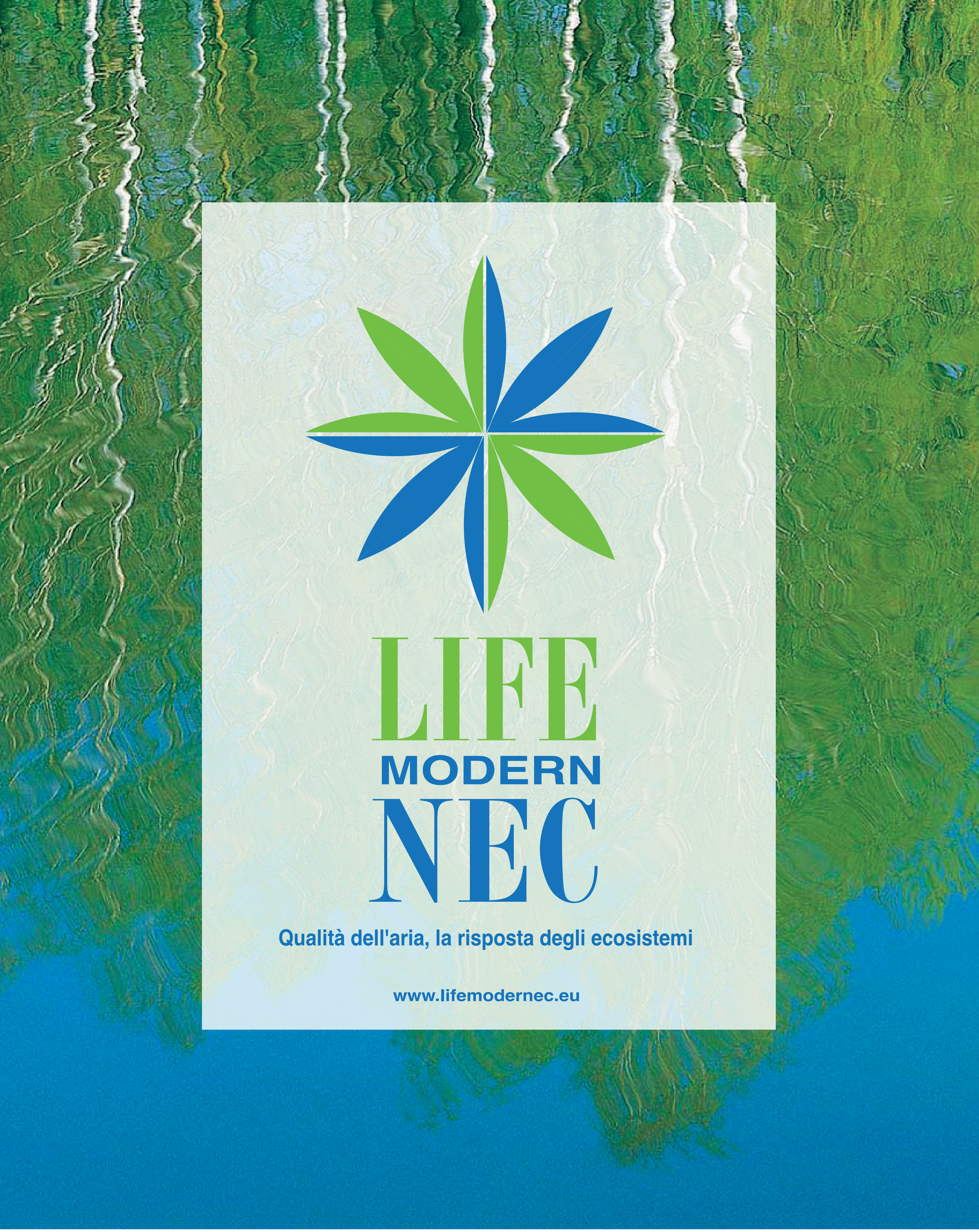

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DELIVERABLE ACTION B.2

Definition of a new set of indicators for the study of atmospheric pollution impacts on ecosystems.

Contents

Summary

In Action B2 has been selected a set of new indicators, with attendant protocols to be tested in action B3, to each protocol a manual for field activities will correspond (B4). Each new indicator, excluding the one related to Remote Sensing, will need on-field data collection at existing and new monitoring sites selected in action B1. For these purposes the UNICAM team has coordinated the partners involved in action B2 and indicators were selected analyzing the research and experiences according to the needs highlighted in A2.

17 new indicators were selected and the related biodiversity and visibility protocols produced.

1. Introduction

The current strategy adopted by Italy to meet the requirement of the NEC Directive mainly follows the protocols developed by the International Cooperative Programs (ICPs), running under the Convention on Long-Range Transboundary Air Pollution (CLRTAP); in particular, ICP-Waters for freshwater ecosystems and ICP-Forests for terrestrial ecosystems. In this context, the main result of the LIFE MODERn (NEC) project is the qualitative and quantitative improvement of the dataset at NEC representative sites to fully meet the requirements of art. 9 of NEC Directive. This will be achieved by increasing the number of monitoring sites and indicators of the Italian NEC network.

The goal of the action B2 is to select new indicators, according to the needs highlighted in the action A2, with attendant monitoring protocols to be tested in action B3. Each protocol will correspond to a manual (action B4) for the on-field operations, each manual will be tested and set during the training and intercalibration on-field courses. Protocols and manuals are the basis for the training and intercalibration of survey teams, concurring synergistically to: A. the reduction of systematic error in long-term and large scale terrestrial surveys;

B. the improvement of Italian NEC monitoring strategy efficiency.

In this context a critical review of contents produced in action A1 and A2 were initially performed by the UNICAM coordination team, three discussion and coordination video conferences were organized among the partners involved focusing on potential and needs of a new set of indicators. Seventeen new indicators have been subsequently selected and the related biodiversity and visibility monitoring protocols have been produced. The only indicator with no protocol related is the one connected to Remote Sensing because no field operations are planned. All the new seventeen indicators will be tested (action B3) with surveys in the existing and new monitoring sites.

2. The set of new indicators

Table 1. Table listing the new indicators, the macro category to whom they belong (following B2 action), the corresponding protocol type, and the related responsible partner.

Indicators	**Indicators macro category**	**Protocol type**	**Partner**
Visibility Index (Bext)	Air quality	Visibility	CUFAA – Cristiana Cocciufa, ENEA - Ettore Petralia
Specific Leaf Area (SLA)	Plant functional and community-based assemblages in forest ecosystems	Biodiversity	UNICAM - Marco Cervellini, Roberto Canullo, Giandiego Campetella, James Tsakalos - CNR IREP - Bruno De Cinti
Compositional Diversity (CD).	Plant functional and community-based assemblages in forest ecosystems	Biodiversity	UNICAM - Marco Cervellini, Roberto Canullo, Giandiego Campetella, James Tsakalos
Functional groups (e.g., Ellenberg)	Plant functional and community-based assemblages in forest ecosystems	Biodiversity	UNICAM - Marco Cervellini, Roberto Canullo, Giandiego Campetella, James Tsakalos

Photosynthetic efficiency - plant vitality	Plant functional and community-based assemblages in forest ecosystems	Biodiversity	UNIFI-DAGRI - Filippo Bussotti e Martina Pollastrini
Abundance (total and relative) and diversity of macroinvertebrate and diatom taxa	Macroinvertebrates and diatoms (freshwaters)	Biodiversity	CNR-IRSA, Simona Musazzi, Aldo Marchetto, Angela Boggero e Michela Rogora
Lichen functional Diversity - Functional groups: growth form Percentage contribution to the Lichen Diversity Value (LDV) of each functional group. 1) %LDV foliose lichens; 2) %LDV fruticose lichens; 3) %LDV crustose lichens. Tree level assessment and plot level reporting.	Priority Epiphytic Lichens	Biodiversity	TERRADATA – Giorgio Brunialti e Luisa Frati
Lichen functional Diversity - Functional groups: nitrogen sensitive species (oligotrophic vs nitrophytic species). Percentage contribution to the Lichen Diversity Value (LDV) of each functional group. 1) %LDV oligotrophic species; 2) %LDV nitrophytic species. Tree level assessment and plot level reporting.	Priority Epiphytic Lichens	Biodiversity	TERRADATA – Giorgio Brunialti e Luisa Frati
Viability and conservation assessment of the sensitive species *Lobaria pulmonaria*	Priority Epiphytic Lichens	Biodiversity	TERRADATA – Giorgio Brunialti e Luisa Frati

a rapid biodiversity assessment (rba) method 1) abundance of *Lobaria pulmonaria* (tree and plot level); 2) presence of juvenile thalli (< 2cm); 3) presence of meristematic lobes; 4) presence of lobes with vegetative propagules; 5) presence of fruiting bodies. Tree level assessment and plot level reporting.			
Lichen functional Diversity - guidelines for the assessment of epiphytic fruticose lichen diversity on the ground 1) number of species; 2) species abundance. Plot level assessment.	Priority Epiphytic Lichens	Biodiversity	TERRADATA – Giorgio Brunialti e Luisa Frati
Lichen functional Diversity - guidelines for the assessment of epiphytic fruticose lichen diversity on the ground Concentration of the main metals and trace elements (Al, As, Cd, Cr, Cu, Hg, Ni, Pb, Ti, V, Zn) in foliose and/or fruticose lichens. Plot level assessment.	Priority Epiphytic Lichens	Biodiversity	TERRADATA – Giorgio Brunialti e Luisa Frati
Community analysis: taxonomic diversity of	Faunal diversity	Biodiversity	CNR-IRET - Paolo Colangelo

chiroptera; relative abundance (based on vocalizations)			CNR_IRET - Bruno De Cinti CNR_IRET - Flavia Sicuriello
Community analysis: Acoustic complexity of the community	Faunal diversity	Biodiversity	CNR-IRET - Paolo Colangelo CNR_IRET - Bruno De Cinti CNR_IRET - Flavia Sicuriello
Community Analysis: Taxonomic diversity (Family/Gender Level) Environmental DNA (eDNA); target taxa invertebrates and mammals	Faunal diversity	Biodiversity	CNR-IRET - Paolo Colangelo CNR_IRET - Bruno De Cinti CNR_IRET - Flavia Sicuriello
Community Analysis: Taxonomic diversity (Family/Gender Level) QBS;relative abundance	Faunal diversity	Biodiversity	CNR-IRET - Paolo Colangelo CNR_IRET - Bruno De Cinti CNR_IRET - Flavia Sicuriello
Dates (Day Of the Year-DOY) for phenological metrics (Greenup, Maturity, MaxValue vegetation index, Senescence, Dormancy)	Plant functional and community-based assemblages in forest ecosystems	Biodiversity Remote Sensing (RS) - no protocol has been produced because no field operations are planned	IREA CNR – Daniela Stroppiana
Pollutant retention capability of the soil-forest system	Pollutant fluxes	Biodiversity	UNIFI-DST – Stefano Carnicelli UNIFI-DST – Anna Andretta

3. Visibility Protocol

3.1. VISIBILITY INDEX (Bext)

B2 macro category: Air quality

Partner: CUFAA – Cristiana Cocciufa, ENEA - Ettore Petralia

1. Introduction

Visibility is a measure of how well an observer can view a scene. This includes how far one can see through the atmosphere as well as the ability to see the textures and colors of the scene. Visitors coming to natural areas may sometimes experience vistas obscured by haze, often also caused by fine particles and gaseous air pollution: these particles and gasses impact visibility by scattering and absorbing light in the atmosphere. Although haze does occur naturally, due to local meteorological conditions (e.g. humidity), dust, wildfire smoke, a fundamental component is often represented by air pollutants from anthropogenic sources. For this reason, monitoring of visibility is based on a robust analysis of air quality as well of meteorological conditions at a site. Considering visibility as an ecosystem service, impaired air transparency can heavily affect the fruition of natural areas. Moreover, visibility field campaigns open to the general public have a relevant educational potential, as they can raise awareness of citizens on how air pollution (often invisible) can affect their lives.

The present protocol is a revised short version of the visibility protocol already delivered within Action A2 and used for the visibility monitoring test.

2. Scope and application

The aim of this protocol is to guide the monitoring of air quality in natural, remote areas, through the analysis of: I) atmospheric pollutants present and II) transparency of the air, in order to obtain a measure of the optical vision of the landscape or, in other words, a measure of how much the view is impaired, in the eyes of an observer, by air pollution. This monitoring has the general objective of defining whether and which emission sources of anthropic origin can lead to a reduction in atmospheric transparency and decrease the possibility of enjoying landscapes and naturalistic qualities of the identified rural areas; moreover, it can possibly propose processes to reduce the impact of these sources at the measurement site.

3. Objective

The main objective of this monitoring activity is the quantification of a **coefficient (bext)** which describes the extinction of light as a function of various chemical-physical parameters associated with airborne molecules and particles at a natural remote site. Values of bext are then compared with pictures taken by a photocamera pointed towards a landscape mark several kilometers away from the sampling site, in order to investigate correlations of air quality with air transparency and visibility.

4. Location of measurements/assessments/sampling

Monitoring activity takes place at "rural" (R) - "remote rural", i.e. in NON-urban or suburban sites identified according to the definitions of the European Environment Agency, adopted by the National Environmental Protection System (SNPA), for the study of atmospheric pollution. Sites should be more than 50 km away from sources of polluting emissions. Protected areas are preferably selected, when possible.

5. Measurements/assessments/sampling (+QA|QC)

The reference formula for calculating bext is shown below:

$$b_{ext} \approx 2.2 \times f_S(RH) \times [Small\ Ammonium\ Sulfate] + 4.8 * f_L(RH) * [Large\ Ammonium\ Sulfate] + 2.4 * f_S(RH) * [Small\ Ammonium\ Nitrate] + 5.1 * f_L(RH) * [Large\ Ammonium\ Nitrate] * 2.8 * [Small\ Organic\ Mass] + 6.1 * [Large\ Organic\ Mass] + 10 * [Elemental\ Carbon] + 1 * [Fine\ Soil] + 1.7 * f_{SS}(RH) \times [Sea\ Salt] + 0.6 * [Coarse\ Mass] + Rayleigh\ Scattering\ (Site\ Specific) + 0.33 * [NO2\ (ppb)]$$

Samplings, lasting 24 hours, are carried out during an entire calendar year with a frequency of one sampling every three days (122 samplings/year). Four particulate sampling lines are necessary: three sampling lines for the PM2.5 fraction (I. line set up with a Teflon filter for the determination of the mass concentration of the particulate and of metals and trace elements, II. line with quartz filter for the analysis of EC and OC and III. a line in which a denuder is inserted to eliminate substances during sampling interferents in the gas phase, prepared with a Nylon or Teflon filter for the measurements of anions and cations) and IV. a PM10 line for the subsequent gravimetric analysis. The measurement of the NO2 concentration is obtained using a monitor for the quantification of NOx (continuous sampling). The Rayleigh scattering coefficient should be determined as the annual average of the data collected by a nephelometer placed on the measurement site but, as a first approximation, this coefficient can also be replaced by a reference value calculated in a similar site in terms of altitude and average annual temperature. Air relative humidity (RH) is delivered by a weather station installed close to the air sampling shelter. In particular the weather station measures: air temperature and humidity, precipitation, wind speed and direction, solar radiation (continuous sampling). Pictures of the target landscape mark are taken by a camera (medium photographic zoom), with one shot every 5 minutes on PMs sampling days.

6. Data handling

The determination procedure of the bext coefficient involves sampling on various types of PM2.5 and PM10 filters (teflon and quartz filters); the samples are then managed in the laboratory for the chemical and physical analyses. During the post-sampling phase until laboratory analyses, filters are stored refrigerated. NOx, meteo data and pictures are managed by remote connection.

7. References

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Malm W.C., Sisler J.F., Huffman D., Eldred R.A. and Cahill T.A., 1994. Spatial and seasonal trends in particle concentration and optical extinction in the United States. J. Geophys. Res. 99: 1347–1370.

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8. Annexes

The annexes should or however we deem necessary also include the following documents (to be produced after initial discussion and at starting of sampling):

1) Sampling timetable
2) Worksheet on activities and anomalies at the visibility sampling site

4. Biodiversity Protocols

4.1. SPECIFIC LEAF AREA (SLA)

B2 macro category: Plant functional and community-based assemblages in forest ecosystems

Partners: UNICAM - Marco Cervellini, Roberto Canullo, Giandiego Campetella, James Tsakalos - CNR IREP - Bruno De Cinti

1. Introduction

Functional traits are features of individual organisms related with their fitness and/or their effect on ecosystem processes, their use is therefore promising to address a variety of ecological questions from a generalized point of view (TraitDivNet 2022). Plant traits (morphological, physiological, phenological) are indicative of ecological strategies and have the potential to build predictive relationships with the environment as the quantification of a wide range of natural and human-driven processes, including alterations in biogeochemical processes and vegetation–atmosphere interactions (Pérez-Harguindeguy at al. 2016). Indeed plant traits could be considered as valid indicators to monitor general ecosystem dynamics and vegetation community responses to environmental changes (e.g., biogeochemical cycles) as opposed to more traditional diversity measurements (e.g., richness indexes) (Standish et al., 2014; Brudvig, 2017). Particularly, Specific Leaf Area trait (SLA) is the one-sided area of a fresh leaf, divided by its oven-dry mass and is related to leaf Nitrogen (N) concentration (Pérez-Harguindeguy at al. 2016).

2. Scope and application

SLA therefore is a trait to be used as an indicator suitable to represent the complexity of air pollution impacts on forest understory community-based assemblages.

The protocol is aimed to gather organic material to assess the relationship between atmospheric pollution and its influence on SLA.

3. Objective

Will be selected the fully expanded and hardened leaves from adult plants.

4. Location of measurements/assessments/sampling

The location of the surface sampled corresponds to the ICP Forests/CONECOFOR 50 m x 50 m plots present in each 10 forest sites selected in action B.1.

5. Measurements/assessments/sampling (+QA|QC)

The most 5 abundant species at plot level (considering both herbaceous and woody species) will be sampled. Leaves will be collected in 5 different individuals of each species (1 leaf per each individual, in total 25 leaves from 5 species). Leaves sampled will be stored on the field in a proper container able to maintain air humidity.

6. Data handling

Measurements and data handling will be done in laboratory

7. References

TraitDivNet (2022) version 24

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8. Annexes

The following contents will be part of manual (B4 action):

1) Guidelines for QA/QC field survey
They should contain measures to minimize variability of errors in data collection among surveyors
2) Notes to avoid disturbances to other field observations (this note will be agreed upon with all teams conducting field observations)

4.2. COMPOSITIONAL DIVERSITY (CD)

B2 macro category: Plant functional and community-based assemblages in forest ecosystems

Partners: UNICAM - Marco Cervellini, Roberto Canullo, Giandiego Campetella, James Tsakalos - CNR IREP - Bruno De Cinti

1. Introduction

Assessment of biological diversity faces the needs of one of the six essential criteria for sustainable forest management (“C4: Maintenance, conservation and appropriate enhancement of biological diversity in forest ecosystems”; MCPFE 2003). Particularly, among the components of forest biodiversity, vegetation is the foundation of ecosystem functioning and primary production, thus ecosystem services. Furthermore, vascular understorey species, while accounting for less than 1% of forest biomass, may constitute more than 90% of plant diversity (Gilliam 2007). Thus, the study of plant diversity can provide information on the status of the ecosystem, the impact of complex disturbance regimes, and offers a baseline for assessment of temporal variability responses (Canullo et al. 2013). The monitoring of plant diversity as a response factor is usually performed at stand-scale, on fixed sized surfaces and frequently based on textural elements (i.e. species richness and relative abundance). In reality, forest dynamics takes shape through structural spatio-temporal variations at different biological, temporal and spatial scales (changes in species abundance, heterogeneity, spatial dependence, mobility, coexistence mechanisms, etc.). Such complex multivariate interactions can be appropriately described by scale-dependent biocomplexity indicators. In this respect, to define a relative “dynamic” status of forest stands we will use a new indicator called “compositional diversity” (CD). It represents an effective “coenostate” descriptor providing the spatial variability of species combinations. The model is based on the information theory (JNP models, Juhász-Nagy and Podani 1983), and its inherent property is that it is relevant only if determined for a series of increasing sampling unit size, where scale is no longer a constraint, but part of the main issue of the study (Wildi et al. 2004, Tsakalos et al. 2022).

2. Scope and application

CD was tested and proved to be applicable in a wide range of vegetation types including forests understory, old fields and abandoned grasslands, tall- and short-grass steppes, mountain grasslands and semi-desert communities. Furthermore, CD resulted to be sensitive in detecting spatio-temporal changes on species assemblages (Bartha et al. 1998, 2004; Bartha 2008, 2014 and literature therein; Campetella et al. 1999, 2004, 2014).

The protocol is aimed to assess and to monitor the fine-scale plant assembly patterns on defined conditions, as resulting from complex coexistence mechanisms of the plant vascular understory species (growing on mineral soil).

3. Objective

We will detect the presence and absence of the plant vascular species to calculate the new indicator. This allows a statistics of species combination and association to be assessed across a large range of scales (sequentially increasing sampling lengths) by applying different randomization techniques and building neutral models for statistical reference (Bartha and Kertész 1998, Campetella et al. 1999). Finally, calculation of the CD will define the synthetic biodiversity status of forest stands and its changes.

4. Location of measurements/assessments/sampling

The location of the surface sampled corresponds to the ICP Forests/CONECOFOR 50 m x 50 m plots present in each 10 forest sites selected in action B.1.

5. Measurements/assessments/sampling (+QA|QC)

Within each plot:

1. For Transect survey
    - A 100m long circular transect composed by 1000 small contiguous Sampling Units (SU: 10 cm x 10 cm micro-squares) will be arranged, in a way that it will cross (proportionally) all patch types of the above ground plant community heterogeneity, including vegetation cover discontinuities;
    - at each 10 cm, the corresponding SU will be materialized;
    - a progressive number of each SU will be reported on the field form;
    - for each SU each taxon must be attributed to a terricolous vascular plant species (*Genus* and *species* binomial) and then reported on the field form using the corresponding progressive number. All the plant species "rooted" or "non rooted" in the SU (the latter is the case in which organs, individuals or branches from outside the SU hang in the SU) will be considered. Other organisms, namely mosses and lichens, if any, must be considered only as groups (not species distinction);
    - to each species will be associated an univocal code and this will represent the species on the field form.

2. For Plot survey
    - A list of the vascular plant species "rooted" or "non rooted" in the plot (the latter is the case in which organs, individuals or branches from outside the plot hang in the plot) will be compiled;
    - % cover of tree canopy, shrub layer, herb layer, mosses and lichens, bare soil, litter and any local feature or disturbance will be recorded and reported on the field form;

Plot survey will follow the Transect survey, or can be done parallel by another team.

6. Data handling

Field data entry will be done using a paper form, then the survey teams will transcript to an electronic sheet each field form. Plot and transect datasets will be transcripted on separate files.

7. References

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8. Annexes

The following contents will be part of manual (B4 action):

1) Guidelines for QA/QC field survey
They should contain measures to minimize variability of errors in data collection among surveyors
2) Notes to avoid disturbances to other field observations (this note will be agreed upon with all teams conducting field observations)

4.3. FUNCTIONAL GROUPS (FG)

B2 macro category: Plant functional and community-based assemblages in forest ecosystems

Partners: UNICAM - Marco Cervellini, Roberto Canullo, Giandiego Campetella, James Tsakalos - CNR IREP - Bruno De Cinti

1. Introduction

Defining the abiotic site conditions from environmental relationships of plant species has a long research tradition. The initial core study belong to Ellenberg (1974) that calculated indicator values for Germany, based on field observation and partly on measurements, for seven abiotic environmental variables: light, temperature, continentality, moisture, soil reaction, nutrient (nitrogen) content, and salinity. Ellenberg's approach was then adopted to create several regional systems of indicator values for other parts of Europe (Tichý et al. 2022). Tichý et al. (2022) is the most recent research work published on this topic, the authors have compiled a harmonized data set of vascular plant indicator values suitable for a large part of Europe. Particularly in this study the number of species selected to calculate the indicators for the Italian territory corresponds to 66% of the total species pool investigated. Thus, the published dataset with Hellenberg values harmonized at EU scale could be used to define a community-based indicator for the assessment of effects of atmospheric pollution on plant diversity.

2. Scope and application

The “functional groups” indicator is a tool for an indirect calculation of environmental conditions based on the selection of suitable and available environmental Hellenberg variables.

3. Objective

To calculate the community means, based on species cover-abundances per plot, of hellenberg value.

4. Location of measurements/assessments/sampling

The location of the surface sampled corresponds to the ICP Forests/CONECOFOR 50 m x 50 m plots present in each 10 forest sites selected in action B.1.

5. Measurements/assessments/sampling (+QA|QC)

All the measurements to calculate the indicator will be carried out in the laboratory. Consequently all the measurements/assessments/sampling are not foreseen by this protocol aimed to describe the actions to be carried out in the field

6. Data handling

Data handling actions related to the "functional group" indicator are the same of those reported in the Data handling section - "Plot survey" subsection of "compositional diversity"protocol

7. References

Tichý, L., Axmanová, I., Dengler, J., Guarino, R., Jansen, F. & Midolo, G. et al. (2023) Ellenberg-type indicator values for European vascular plant species. Journal of Vegetation Science, 34, e13168. Available from: https://doi.org/10.1111/jvs.13168

8. Annexes

The following contents will be part of manual (B4 action):

1) Guidelines for QA/QC field survey
They should contain measures to minimize variability of errors in data collection among surveyors
2) Notes to avoid disturbances to other field observations (this note will be agreed upon with all teams conducting field observations)

4.4. PHOTOSYNTHETIC EFFICIENCY - PLANT VITALITY

B2 macro category: Plant functional and community-based assemblages in forest ecosystems

Partner: DAGRI - University of Firenze - Italy - Filippo Bussotti and Martina Pollastrini

1. Introduction

The content of chlorophyll, together with the efficiency of photosystems (to be measured through chlorophyll a fluorescence), are indicators of plant vitality, and gives a comprehensive picture of the overall status of a photosynthetic organism. Chlorophyll content and fluorescence can be measured in the field by mean of nondestructive methods, with commercial chlorophyll meters and fluorimeters. The parameters assessed gives information on the “potential” photosynthetic efficiency, but not on the “real” photosynthetic performance, so the connection with the net photosynthesis (*P*n) is elusive, but not to be excluded.

The chlorophyll a fluorescence (ChlF) is a mechanism of energy dissipation (non-photochemical de-excitation) in photosynthetic organisms. ChlF may be “passive”, emitted by leaves exposed to the solar radiation and, in this form, is assessed by mean of remote sensing survey (Sentinel 3 programme). In field application ChlF is assessed in the “active” form, i.e., emitted by dark adapted leaves submitted to a saturating (actinic) light.

The analysis of ChlF in dark adapted samples (PF) allows to measure the minimum (F_0) and maximum (F_M) fluorescence emission. This measurement takes less than 1s. The kinetic of the ChlF emission between F_0 and $F_{M,}$ graphically represented by ChlF transient, is analyzed by means the called JIP test that allows to collect a constellation of time-dependent parameters collectively (Strasser et al., 2000; 2004; Tsimilli-Michael and Strasser, 2008).

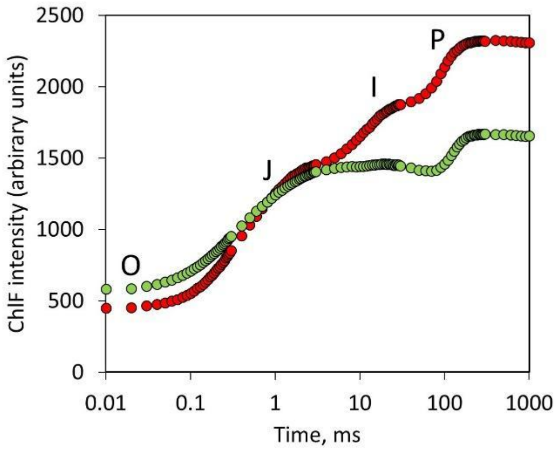

Fig 1. – Examples of OJIP transients for unstressed (red) and stressed (green) plants. The rising OJIP curves are induced by a short pulse (1 s of duration) of saturating red light (650 nm). Plotted on a logarithmic time scale, the ChlF transients show a polyphasic shape with the time. The label O refers to the initial fluorescence level (F_0); J (2 to 3 ms), I (30 ms) and P (500-800 ms - 1s) are, respectively, intermediate and the peak levels of the fluorescence emission. The latter indicates the highest, or maximal, fluorescence intensity (F_M), when saturating light is applied to the leaf.

2. Scope and application

The measurement of ChlF parameters and kinetics of ChlF emission can provide useful information about the state and functioning of the photosynthetic apparatus, with particular attention to the photosystems (PSII and PSI). ChlF is a widely applied technique to assess plant responses to environmental factors. There are two main scientific approaches: the modulated and the direct (or prompt) ChlF. The modulated ChlF is based on the measurement of fluorescence emission by light exposed samples; the prompt fluorescence (PF) is applied on dark adapted samples and needs just one second to take the measurements, allowing to have many samples measured in a short time.

3. Objective

In the context of the ICP Forests monitoring programme the assessment of chlorophyll content and fluorescence is recommended to follow the possible physiological impact over time of environmental conditions and climatic events, as well as to explore possible relationships with deteriorating crown conditions and foliar nutrition.

4. Location of sampling and measurements

The assessment of chlorophyll related parameters can be carried out at the same areas and sampling time of the action related to “nutrient” assessment.

5. Sampling and measurements

5.1. *Sampling*

The preferable sampling period is between mid-June and mid-August of each year, in the presence of mature leaves and before the onset of the leaf senescence.

The leaf sampling can be carried out by means of tree climbers, extension loppers and gun shooters according to the height of the trees, the stand structure, and the operational conditions in each forest plot. Branches 40-50 cm long with attached leaves should be sampled in the highest southern exposed part of the crown, in the upper portion (i.e., top leaves, fully exposed to sun).

Chlorophyll content can be measured immediately after sampling by chlorophyll meters.

For the measurement of chlorophyll fluorescence, the cut branches should be immediately placed in hermetic plastic bags and humidified to avoid the dehydration. The bags with the samples are then placed in insulated boxes keeping the samples at constant temperature.

5.2. *Measurements*

The value of many fluorescence parameters varies according to the time of the day, due to the duration of the leaf sunlight exposure. At midday we have a depression of F_V/F_M whereas I-P phase parameters increase from the beginning of the morning to noon. The dark-adaptation of leaves prior to the prompt ChlF measurements is usually performed using leaf clips, for a time of 20–30 minutes. In this way the dynamic photoinhibition of leaves is eliminated, but not their potential chronic photoinhibition. Dark-adaptation of the samples for longer periods (at least four-five hours) is necessary to effectively reduce most components of the photoinhibition of the leaves, thus reducing the margin of error in the comparison between the ChlF properties of leaves.

The measurement of ChlF parameters, by portable fluorimeters, can be done at the end of the sampling day, in a dark room. For a robust sampling, it is recommended to make at least 15-20 measurements per tree, to make up for any wrong or failed measurements.

Needles of conifers must be grouped to fill the hole of the clips. In evergreen trees, we suggest measuring only the last year of leaves or, alternatively, data from leaves with different ages should be reported separately.

5.3. *Instruments*

Chlorophyll contents

- SPAD-502 (Konica Minolta, Japan)
- CL-01 Chlorophyll Con-tent System (Hansatech Ltd., Norfolk, UK)

- CCM-200 plus and CCM-300 (ADC)
- atLEAF CHL chlorophyll meter (FT Green LLC®, USA)
- Dualex® and Multiplex® (Force-a, Paris, France)

Chlorophyll fluorescence

- HandyPea (Hansatech Ltd., Norfolk, UK)
- PocketPea (Hansatech Ltd., Norfolk, UK)
- FluorPen (Photon System Instruments, Drásov, Czech Republic)
- MiniPAM (Walz, GmbH, Effeltrich Germany) (only for Fv/Fm)

5.3. *Parameters*

Chlorophyll content

The parameter assessed consists in a scale of arbitrary units (different in the various instruments) proportional to the content of chlorophyll and nitrogen.

Chlorophyll fluorescence

The most popular and applied parameter, calculated from F_0 and F_M, is the maximum quantum yield of a sample in the dark-adapted state (**$F_V/F_M = [F_M - F_0]/F_M$**), i.e., the maximum capacity of PSII to trap photons. F_V/F_M is considered the most suitable proxy to assess the efficiency of the PSII.

Based on the analysis of large datasets, we suggest using **F_V/F_M (TR_0/ABS)** and **Ψ_{Eo} (ET_0/TR_0)** as parameters to evaluate PSII functionality and **I-P phase** to assess that of PSI. Although very popular in experimental research and in small field surveys, we don't recommend the use of the Performance Indices (**PI_{ABS}** and **PI_{TOT}**) in monitoring activities, since they have large variability and can be affected by instrumental bias.

ChlF parameters are dimensionless. Raw data are expressed as arbitrary units (a.u.). Parameters can be expressed as ratios or arbitrary units. Only ratio parameters can be easily comparable between different instruments.

Selected JIP-test parameters and their physiological significance.

Parameter	Physiological significance	Unit	Definition and further physiological details on the parameter
$F_V/F_M = TR_0/ABS$ $[F_M - F_0]/F_M$	PSII efficiency	ratio	Trapping probability, or maximum quantum yield of primary photochemistry of a dark-adapted leaf. it is the probability that an absorbed photon will be trapped by the PSII reaction centers.
$\Psi_{Eo} = ET_0/TR_0 = 1 - V_J$	PSII efficiency	ratio	Probability that a photon trapped by the PSII reaction center enters in the electron transport chain.
ΔV_{IP} = I-P phase = $1 - V_I$	PSI efficiency	ratio	The amplitude of the ChlF emission transient between I-to-P time-phase (i.e. between 30 millisecond the time of the maximum ChlF value). It is the relative contribution of the I-to-P phase to the whole ChlF emission transient (OJIP transient). It is a proxy of the PSI

			concentration in the photosynthetic apparatus.
PI_{ABS}	Overall PSII efficiency	a.u.	Performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors.
PI_{TOT}	Overall PSII and PSI efficiency	a.u.	Performance index (potential) for energy conservation from the photons absorbed by PSII to the reduction of PSI end-electron acceptors.

5.5. *Possible problems related to measurements*

Chlorophyll content

The instruments read the transmitted light from a fixed surface, and the content of chlorophylls is related to the thickness of the leaf. Therefore, the values provided depend on the foliar anatomy and different species are difficult to be compared. To transform the content in concentration it is necessary to consider the foliar thickness.

The units provided by the measurements are arbitrary (although proportional to the content of chlorophyll) and differ in the different instruments. It is accepted that the reference is represented by SPAD-502 (SPAD units). The tables for the transformation of the instrumental readings with SPAD units are provided by the manufacturer.

Although in literature curves for the transformation of SPAD units in chlorophyll content are available, it is recommended to construct calibration curves for the different species, by comparing the instrumental readings with laboratory analyses.

Chlorophyll fluorescence

The absolute values of F_0, F_M and all the time-dependent points of the ChlF emission transient are expressed in arbitrary units and their raw values are dependent from the technical characteristics and settings of the instruments used and by the intensity of the actinic light applied to the sample, by the lamp incorporated in the instrument. Therefore ratios (as F_V/F_M) and normalized parameters (from the normalized ChlF emission transient) are more robust and comparable among them than the raw original data. Original data can have high variability and little comparability among different instruments and field operators. The initial slope of the ChlF transient (M_0) may also be affected by the instrumental characteristics. ChlF parameters are also influenced by the thickness of leaves, their chlorophyll content, the presence of necrosis or other foliar damages, as well as by the position of the leaf clip on leaf lamina (for ex., above the veins) and the intrinsic intra-leaf variability.

Recent research evidence that the value of F_0 is affected by a relevant contribution of the fluorescence from PSI (about 30%). F_V/F_M is therefore underestimated using the commercial devices that are based on the detection of the fluorescence by PSII. However, we assume that the proportionality and the significance of the differences between the values of ChlF parameters, measured on different samples, is maintained.

5.6. *Quality Assurance*

Chlorophyll meters are “autocalibrated” and do not need calibration by manufacturers before the monitoring campaigns.

Fluorimeters must be calibrated by the manufacturer before any field campaign.

It is advisable to have a “reference team” for intercalibration of field team members and the correspondence of field readings with actual concentrations.

Joint sessions of intercalibration between the operators are recommended.

<u>Recommended settings for HandyPea</u>

Duration: 1s

Lamp intensity: 3000

Gain: 0.75 (the reduction of the gain <1 is necessary to avoid values over the range in thick leaves)

6. Data handling

After the control of the dataset to eliminate the wrong data (anomalous curves) and failed measurements, the values of the parameters, expressed as average per tree, are organized in Excel sheets with the following information:

- Country
- Plot
- Tree number
- Species
- Leaf age (in evergreen trees)
- Date
- Duration of dark adaptation (for chlorophyll fluorescence)
- Instrument

Raw data, as they are downloaded from the device, are also encouraged to be provided (one file per tree, jointly with the above listed information).

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4.5. ABUNDANCE (TOTAL AND RELATIVE) AND DIVERSITY OF MACROINVERTEBRATE AND DIATOM TAXA

B2 macro category: Macroinvertebrates and diatoms (freshwaters)

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1. Introduction

Macroinvertebrates and diatoms have been considered as key groups for freshwater monitoring programs since the ‘70s (Descy and Coste, 1991; Rosenberg and Resh, 1993). Undemanding nutritional habits, simple structure, fast growth and both sexual and asexual reproduction make diatoms extremely adaptable to various environmental conditions, resulting in their high survival ability and biodiversity (Krivograd Klemenčič and Toman, 2010). Macroinvertebrates include immature and adult stages of many different groups of invertebrates colonizing all types of water bodies. Their distribution is mainly influenced by substrate, water depth, temperature, chemistry, food availability, and they act as a crucial link in the food webs by connecting organic matter resources with lower and higher trophic levels (Hauer & Resh, 2006; Boggero et al., 2018). Diatoms are considered good indicators of freshwater quality, as their assemblage changes in relation with e.g., trophic condition, oxygen saturation, temperature, pH (Bennion et al., 2014; Kelly et al., 2012).
For these reasons, macroinvertebrates and diatoms have been selected as biological indicators to assess the ecological status of freshwater bodies within the EU Water Framework Directive (WFD, 2000/60/EC) (European Commission, 2000). They are indeed sensitive to a wide range of anthropogenic disturbance, including eutrophication, organic pollution, trace metals, and climate change (Baker et al., 2021; Karst-Riddoch et al., 2005; Svitok et al., 2021). Due to their sensitivity to acidification, they have been also adopted as indicators in research studies and monitoring programs aiming to assess the response of ecosystems to the deposition of atmospheric pollutants (Battarbee, 1994; Brakke et al., 1994). Furthermore, both groups are considered in the ICP WATERS Manual as possible indicators for the biological monitoring of atmospheric pollution effects on freshwaters (ICP Waters Programme Manual 2010).
Beside the possible effects of atmospheric pollution on the assemblage composition, climate change can also impact macroinvertebrates and diatoms, for instance by i) shifts towards euryhaline and aerophilic species, ii) losses of sensitive species, and ultimately iii) alterations of biodiversity (Sommaruga, 2015; Rühland et al., 2015; Hotaling et al., 2017). Hence, collecting new data will represent a benchmark to evaluate the combined effects of multiple stressors (e.g., acidification, nitrogen enrichment, climate change) on sensitive habitats.

2. Scope and application

Despite the documented effectiveness of macroinvertebrates and diatoms as indicators of atmospheric pollution and climate change impacts on freshwater ecosystems, their monitoring at ICP WATERS, and more generally, at sensitive sites in Italy have been rather limited (Marchetto et al., 2004, 2009). The long-term response of freshwaters to changing deposition have been mostly evaluated through chemical indicators (e.g., pH, alkalinity, base cations, N compounds) and biological sampling and analyses have been performed on a few occasions, mainly in the framework of EU-funded projects (Marchetto et al., 2009; Fureder et al., 2006). The establishment of the NEC Italy network and the launch of the LIFE MODERn NEC project represent opportunities to extend the monitoring also to biological indicators. The biological surveys will be always performed in association with the evaluation of the chemical status of the water bodies, based on established indicators (pH, alkalinity, major ions, nutrients, trace metals).

3. Objective

Macroinvertebrates and diatoms have been selected as indicators to be used at the freshwater sites based on the provision of the NEC Directive, Annex V, and on previous experience on the use of these taxa for biological monitoring on high-altitude lakes and more generally on sites sensitive to atmospheric pollution (Boggero et al., 2019). The adopted protocols are strongly based on those used within the ICP WATERS or developed during previous projects (NIVA, 1987; ICP waters Programme Centre, 1996, 2010; Boggero et al., 2011); however, they will be adapted to the specific characteristics of the project sites.

4. Location of measurements/assessments/sampling

The sampling will be performed at the selected sites by collecting samples from the lake/stream shores. The number of samples will be site-specific (linking site characteristics such as area/length, habitat types, substrate type to the assemblage structure of both groups). Samples for chemical analyses will be always collected in association with the biological sampling.

5. Measurements/assessments/sampling (+QA|QC)

Macroinvertebrates will be sampled using a handle-net (250 µm mesh aperture) along the littorals, at a maximum depth of 0.5 m, to obtain semi-quantitative samples. At each site, the substrate or the habitat type present (mud, gravel, cobbles, pebbles or rocks, vegetation cover or not), known to control macroinvertebrate abundance and distribution (Abraham et al., 1999; Jyväsjärvi et al., 2013), will be examined by dragging the substrate over a length of 1 m. Three replicates (or a different number, according to the site characteristics) to capture random biological variation, will be taken.
Samples will be sorted into major macroinvertebrate groups using a stereo-microscope (Leica M125, magnification 80x), and preserved in 80% ethanol for subsequent taxonomic identification. Most of the groups will be possibly identified to species level by preparing slides of sexually mature Oligochaetes, and of immature stages of Chironomids. No adults will be captured because they are

usually rare and their capture involves the use of different tools. Slides will be observed under a Zeiss microscope (1000x). Genus or family level of identification will be chosen when poor conservation status of specimens (injury caused by friction) or the presence of juveniles reduce the likelihood of identification to species level. The identification will be based on specific taxonomic keys: Timm (2009) and Schmelz and Collado (2010) for the oligochaetes; Wiederholm (1983, 1986) for the Chironomids and several guides with national distribution related to the remaining groups of macroinvertebrates (AA.VV., 1977-1985). Relative abundances (%) will then be estimated.
Epilithic diatoms will be sampled on cobbles of appropriate size (mesolithal), preferably free of filamentous algae. At least 5 cobbles will be collected from the littoral zone of each site, far from eventual inflow influence. The top surface of each cobble will be brushed with a clean toothbrush to remove the biofilm (Kelly *et al.*, 1998; CEN, 2003). In the laboratory, the samples will be treated with hot hydrogen peroxide and hydrochloric acid following standard procedures (Battarbee *et al*., 2001), and finally mounted using Naphrax on permanent slides for species identification (Zeiss Axiolab, magnification 1000x). The taxonomic recognition is based on Krammer and Lange-Bertalot (1986-1991), Krammer (2000; 2002; 2003), Lange-Bertalot (2001), Lange-Bertalot et al. (2011), Cantonati et al. (2017) For each sample, a minimum of 400 valves will be identified and results expressed as relative abundances (%) (; Battarbee *et al.*, 2001).
For both groups, an analysis of the assemblage structure, diversity, and abundance of the different taxa or functional groups will be performed in relation to their respective tolerance/sensitivity to pH (acidification), nitrogen compounds and climate-related variables (e.g. temperature) For macroinvertebrates, the following indices will be tested: 1. Raddum 988 (Raddum et al., 1988); 2. Raddum 1990 (Fjellheim & Raddum, 1990); 3. NIVA (Bækken & Kjellberg, 2004); 4. $AWIC_{fam}$ (Davy-Bowker et al. 2003, 2005); 5. $AWIC_{sp}$ (Davy-Bowker et al. 2003); 6. Braukmann (Braukmann & Biss, 2004); 7. LAMM (McFarland et al. 2010); 8. TL (Hämäläinen & Huttunen, 1990). For diatoms, beside the traditional diversity index, species groups will be considered following the new autoecological classification for acidity and nitrates proposed by 1. Carayon et al. (2019). 2. Functional Groups (Rimet et Bouchez 2012). The application of other indices, based on species groups, will be potentially evaluated during the project.

6. Data handling

Field data will be collected (e.g., water temperature, habitat description and substrate type through visual survey), then reported in electronic spreadsheets. Species list will be also reported in excel spreadsheets. Specific software packages will be used to compute indices, with a preference for open-source systems (e.g., R).

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4.6. PRIORITY EPIPHYTIC LICHENS: SELECTION OF NEW INDICATORS OF AIR POLLUTION IN FOREST ECOSYSTEMS

B2 Macro Category: Priority Epiphytic Lichens

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1. Introduction

The activities of Actions A1 and A2, carried out respectively to explore the recent literature and to review the current monitoring protocols, provided a useful starting point to improve the knowledge on the topic of epiphytic lichens as indicators of air pollution.
This allowed us to identify new potential indicators to be used within the NEC network, among those more closely related to pollution response, and with relatively easier and faster assessments.

Table 1 summarizes the list of the proposed indicators with their features and relations with Action A1 and A2. The new indicators, to be tested in Action B3, are briefly defined below:
1) Functional groups: growth form.
2) Functional groups: nitrogen sensitive species (oligotrophic vs nitrophytic species).
3) Sensitive species *Lobaria pulmonaria* viability and conservation assessment.
4) Epiphytic fruticose lichens on the ground.
5) Bioaccumulation of potentially toxic elements (metals and trace elements) in foliose and/or fruticose lichens.

Indicators 1) and 2) concern insights and improvements into the processing and interpretation of data deriving from the application of the ICP Forests manual Part VII.2 (Stofer et al. 2016), which is already applied in current sites of the Italian NEC network.
Starting from lichen diversity data (LDV - Lichen Diversity Value) and referring to the response of lichen functional traits, it will be possible to obtain more targeted information on air pollution.

Indicators 3) and 4), with their relevant variables, can be obtained from the adoption in the field of rapid biodiversity methods, which can be applied even by personnel who are not experts in lichen taxonomy, but who are trained on specific field guidelines.

Indicator 3) is based on field surveys of an indicator species that is easy to identify even by non-expert personnel, while indicator 4) concerns the collection of epiphytic fruticose lichens fallen on the ground. The concept at the basis of this method stands on the fact that the specimens collected by trained personnel will be sent to lichen specialists for species identification.

Indicator 5) is based on the known ability of lichens to accumulate potentially toxic elements (such as trace metals and elements) in concentrations that can be correlated with their environmental levels. The recent guidelines developed by ISPRA can be adopted (Giordani et al. 2020). Although testing this method within the LIFE MODERN NEC is not foreseen, during the fieldwork of Action B3, a feasibility study will be conducted to evaluate its applicability in the NEC network forest plots, especially regarding these aspects: i) which species are available; ii) the availability of a sufficient quantity for analyses. For example, among the foliose species, the most represented biomonitors adopted in the papers examined in Action A1 belong to the Parmeliaceae family, such as *Flavoparmelia caperata*, *Parmotrema arnoldii* and *Hypogymnia physodes* (see e.g., Loppi et al. 1998; Loppi and Pirintsos 2003; Jeran et al. 2007, Manninen 2018; Kłos et al. 2018; Benitez et al. 2019; Ancora et al. 2021), while *Pseudevernia furfuracea* and *Usnea* are the mostly used fruticose ones (see e.g., Otnyukova T. 2007; Conti et al. 2009; Cecconi et al. 2018). Further, most of the studies considered 4 to 26 elements, with Cd, Cu, Hg, Mn, Ni, Pb, and Zn being the most commonly analyzed.

As expected from Action B2, we have developed the protocols for the four indicators to be tested during Action B3, to evaluate their effectiveness and the possibility to include them among the monitoring tools of the Italian NEC Network.
If indicators 3 and 4 will prove to be suitable after the testing phase, they could also be extended to Level I CONECOFOR sites, due to their simplified and rapid assessment. This will allow expanding the representativeness of the Italian NEC Network in the near future. The same is true for indicator 5 which, at present, is only being considered for an assessment of its feasibility.
The protocols are reported in separate files.

Table 1 – New indicators proposed to be tested during Action B3, with a reference to the assessed variables and to their relationship with Actions A1 and A2.

Indicator	Variables	Relation with Action A1	Relation with Action A2

Functional groups: growth form	1) %LDV foliose lichens; 2) %LDV fruticose lichens; 3) %LDV crustose lichens. Tree level assessment and plot level reporting.	Several literature studies show that growth forms of epiphytic lichens are suitable indicators of different environmental factors related to air pollution and climate change (e.g., Giordani et al. 2012; Giordani et al. 2014; Geiser et al. 2019).	Not already available in existing protocols for forest ecosystems monitoring. Information can be obtained by the existing papers reviewed in Action A1.
Functional groups: nitrogen sensitive species (oligotrophic vs nitrophytic species)	1) %LDV oligotrophic species; 2) %LDV nitrophytic species. Tree level assessment and plot level reporting.	Several literature studies show that nitrogen depositions affect lichen communities causing a shift from acidophilic to nitrophilous species (e.g., Frati et al., 2007; Giordani et al. 2014; Geiser et al. 2019, 2021).	Not already available in existing protocols for forest ecosystems monitoring. Information can be obtained by the existing papers reviewed in Action A1.
Sensitive species *Lobaria pulmonaria* viability and conservation assessment	1) abundance of *Lobaria pulmonaria* (tree and plot level); 2) presence of juvenile thalli (< 2 cm); 3) presence of meristematic lobes; 4) presence of lobes with vegetative propagules; 5) presence of fruiting bodies. Tree level assessment and plot level reporting.	*Lobaria pulmonaria* large foliose lichen widely adopted as flagship and umbrella species for nature conservation. It is very sensitive to air pollution and in large decline throughout Europe. It is therefore an excellent indicator of air quality and forest continuity (e.g., Nascimbene et al. 2013, 2016; Brunialti et al. 2015).	Not already available in existing protocols for forest ecosystems monitoring. The sampling protocol used by Brunialti et al. (2015) in the old growth forests of the Cilento National Park can be adopted and tested for the NEC network.
Fruticose lichens in the litter	1) number of species; 2) species abundance. Plot level assessment.	Fruticose lichens are particularly sensitive to pollution and climate change, as their large surface area to mass ratios filter moisture and elements from the air (e.g., Knops et al. 1996; Stanton et al. 2014), and they have strongly declined in areas with atmospheric pollution and intensive forestry (see Esseen et al. 2016). This makes this functional group a useful indicator of air pollution and climate change in forest ecosystems (see e.g., McCune 1994; Dettki and Esseen 2003; Lehmkuhl 2004; Nascimbene et al. 2019).	Not already available in existing protocols for forest ecosystems monitoring. Information can be obtained by the existing papers reviewed in Action A1.
Bioaccumulation of potentially toxic elements (metals and trace elements) in foliose and/or fruticose lichens.	Concentration of the main metals and trace elements (Al, As, Cd, Cr, Cu, Hg, Ni, Pb, Ti, V, Zn) in foliose and/or fruticose lichens. Plot level assessment.	Epiphytic lichens have been adopted as bioaccumulators of metal and trace elements in forest ecosystems by several authors (see e.g., Kłos et al. 2018; Benitez et al. 2019; Ancora et al. 2021; Cecconi et al. 2018).	Not already available in existing protocols for forest ecosystems monitoring. The recent guidelines developed by ISPRA can be adopted and tested (Giordani et al. 2020).

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4.6.1. LICHEN FUNCTIONAL DIVERSITY - FUNCTIONAL GROUPS: GUIDELINES FOR THE PROCESSING OF NEW INDICATORS OF AIR POLLUTION IN FOREST ECOSYSTEMS

1. Introduction

Lichen functional traits, such as growth forms, photosynthetic partners, reproductive strategies, and sensitivity to pollutants (e.g., oligotrophic and nitrophytic species), have been extensively studied in the context of forest monitoring (see e.g., Gadsdon et al. 2010; Marmor et al. 2010, Degtjarenko et al. 2018; Geiser et al. 2014; Ellis et al. 2021; Morillas et al. 2021). Most studies suggest that functional diversity can provide additional information to the study of lichen diversity, allowing for early warning responses to forest environmental changes, above all in response to nitrogen compounds which are now the predominant air pollutant (Giordani et al. 2014). Here we propose a protocol to process the data deriving from the application of the ICP Forests manual Part VII.2 (Stofer et al. 2016) in terms of two functional groups: growth forms (foliose, fruticose and crustose lichens) and nitrogen sensitive species (oligotrophic vs nitrophytic species).

Among the main species traits (photobiont, reproductive strategy, growth form), lichen growth form is the most responsive and reliable indicator for evaluating and comparing the responses of epiphytic lichens to climate, human disturbance and stand structure (Giordani et al. 2012).

Similarly, several studies show that nitrogen depositions may increase nitrophilous species in epiphytic lichen communities (e.g., Frati et al., 2007; Giordani et al. 2014; Geiser et al. 2019, 2021). For this reason, an increase in nitrogen deposition in remote environments, such as the forest sites of the NEC network, is expected to cause a shift from communities dominated by acidophilic species (with little need for eutrophicated substrates, and particularly sensitive to the presence of nitrogen compounds), to nitrophilous ones, that are benefited by nitrogen deposition.

2. Scope and application

This manual aims at providing a consistent methodology to process epiphytic lichen diversity data obtained in the context of the NEC Directive Network by means of the ICP Forests field manual (Stofer et al. 2016).

3. Objectives

Starting from the lichen diversity assessment (species richness, abundance and composition), the main objective of this protocol is to consider functional groups to obtain

additional information based on lichen responses to air pollution and climate change, allowing for early warning responses to forest environmental changes.
Specific objectives are defined as follows:

- to assess the relationships among the different growth forms of lichen species on each tree of the sampled plots;
- to assess the relationships between oligotrophic and nitrophytic species on each tree of the sampled plots.

4. Location of measurements/assessments/sampling
Measurements/assessments/sampling are not foreseen by this protocol, which considers only data processing and interpretation procedures.

5. Measurements/assessments/sampling (+QA|QC)
Measurements/assessments/sampling are not foreseen by this protocol, which considers only data processing and interpretation procedures.

6. Data handling
This chapter concerns the lichen diversity data processing, to obtain response variables, based on functional groups, to monitor changes in forest ecosystems induced by air pollution.

According to the ICP Forests Manual (Stofer et al. 2016), for each sampled tree, the Lichen Diversity Value (LDV) is obtained by the sum of the abundance of all lichen species occurring within a 10 × 50 cm observation grid, divided into 5 squares of 10 × 10 cm, placed at each of the four cardinal points of the trunk (N, S, E, W) at a height of 100 cm above the ground. Therefore, the frequency of each lichen species varies between 1 and 20.
Similarly, the LDV of each functional group (e.g., growth forms; sensitive species) is obtained by the sum of all the species belonging to the group occurring within the sampling grid.

6.1 Growth forms
The proportion of morpho-anatomical characters of lichens on a forest tree may represent an interesting tool to monitor the trend in forest ecosystem changes, in relation to air pollution and climate change. In general, foliose and fruticose lichens are more sensitive to air pollution.
For each sampled tree, three response variables based respectively on the three growth forms (foliose, fruticose and crustose lichens) can be obtained:

$$\%LDV_{\text{foliose lichens}} = (LDV_{\text{foliose}}/LDV_{\text{total}}) * 100$$

$\%LDV_{\text{fruticose lichens}} = (LDV_{\text{fruticose}}/LDV_{\text{total}}) * 100$

$\%LDV_{\text{crustose lichens}} = (LDV_{\text{crustose}}/LDV_{\text{total}}) * 100$

At the plot level, results will be presented in the form of mean values of each variable per tree.

6.2 Nitrogen sensitive species (oligotrophic vs nitrophytic species)

The proportion of oligotrophic and nitrophytic species in the lower trunks of forest trees is a suitable indicator of the impact of oxidized and reduced nitrogen compounds (Gadsdon et al. 2010; Cristofolini et al. 2008; Jovan et al. 2012; Pinho et al. 2012a; 2012b).

Definition of sensitive species for nitrogen – Ecological indicator values are 'expert assessments' that qualitatively express the ecological range of species for different factors. In particular, the eutrophication index concerns the tolerance spectrum of each species to the contribution of dust or nitrogen compounds. The ecological optimum of a species can be expressed by a single number of the ordinal scale, or by several values of the index, which then indicate its ecological tolerance spectrum. The ecological indicator values for eutrophication (including deposition of dust and nitrogen compounds) reported in ITALIC 7.0 (Nimis 2022) specify for each species a range on a 5-class ordinal scale, as follows:

1 - not resistant to eutrophication
2 - resistant to a very weak eutrophication
3 - resistant to a weak eutrophication
4 - occurring in rather eutrophicated situations
5 - occurring in highly eutrophicated situations

Based on this classification, two groups of indicator species can be defined:

- **Oligotrophic species**. Lichens with ecological optimum for values 1 or 2 of the eutrophication index, or with a tolerance spectrum between 1 and 2 (species not resistant to eutrophication or resistant to a very weak eutrophication). For each tree, the percentage contribution of these indicator species to the total LDV is obtained as follows:
  $\%LDV_{\text{oligotrophic lichens}} = (LDV_{\text{oligotrophic}}/LDV_{\text{total}}) * 100$

- **Nitrophytic species.** Lichens with a dust tolerance spectrum that does not include values 1 and 2 of the eutrophication index, and which, instead, include classes 4 and/or 5 (occurring in rather eutrophicated to highly eutrophicated situations). For each tree, the percentage contribution of these indicator species to the total LDV is obtained as follows:

$\%LDV_{\text{nitrophytic lichens}} = (LDV_{\text{nitrophytic}}/LDV_{\text{total}}) * 100$

At the plot level, results will be presented in the form of mean values of each variable per tree.

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4.6.2. VIABILITY AND CONSERVATION ASSESSMENT OF THE SENSITIVE LICHEN *Lobaria pulmonaria*: A RAPID BIODIVERSITY ASSESSMENT (rba) METHOD

1. Introduction

The large foliose species *Lobaria pulmonaria* (L.) Hoffm. is very sensitive to air pollution and in large decline throughout Europe. Several studies demonstrated its suitability both as a flagship and as an umbrella species for nature conservation, since it is easy to identify, and it is associated with many other rare or endangered forest dwelling organisms (see e.g., Nilsson et al. 1995; Campbell and Fredeen 2004; Nascimbene et al. 2010, 2013; Di Nuzzo et al. 2022). Further, its assessment can be successfully undertaken by non-expert lichenologists, e.g., by personnel trained in appropriate training courses. These features make it an excellent indicator of air quality and forest continuity, above all for the development of a Rapid Biodiversity Assessment (RBA) method in the context of the NEC Directive Network.

This method, which is based on the study of the viability of the populations of *Lobaria pulmonaria* in forest plots, refers to the sampling protocol of previous studies carried out by Brunialti et al. (2015a, 2015b).

2. Scope and application

This manual aims at providing a consistent methodology for the assessment and monitoring of the viability and conservation of *Lobaria pulmonaria* at the sampling sites of the NEC Directive Network.

3. Objectives

The main objective of this protocol is to assess the viability and the conservation status of the forest-dwelling lichen species *Lobaria pulmonaria* as an indicator of air pollution and climate change.

Specific objectives are defined as follows:

- to assess the occurrence and abundance of *Lobaria pulmonaria* at the tree and plot level;
- to assess its conservation status and health in terms of active growth (presence of meristematic lobes) and dispersion capacity (presence of juvenile thalli, vegetative propagules and fruiting bodies).

The study of the trends in these parameters in the NEC Network sites, is expected to provide early warning responses to forest environmental changes, occurring in the medium- to long-term period.

4. Location of measurements

The assessment of the occurrence and viability parameters of *Lobaria pulmonaria* is carried out on all the trees growing on Level I or Level II plots, independently on the bark-type tree species or on the DBH of the tree individuals.

5. Measurements

5.1 Data collection

This method can be adopted only in the plots where the species is present. In each plot, the presence of *Lobaria pulmonaria* in the lower trunk of all the trees is assessed.
The abundance of *Lobaria pulmonaria* at the plot level, is recorded adopting the following ordinal scale: i) rare: 1-3 trees per plot; ii) sporadic: 4-10 trees per plot; iii) abundant: >10 trees per plot.

The following features are taken into account on the bole of the colonized trees (from 0 to 2 m):

1) abundance of *Lobaria pulmonaria*. Data are recorded adopting the following ordinal scale: i) rare: 1-3 thalli per tree; ii) sporadic: 4-10 thalli per tree; iii) abundant: > 10 thalli per tree.
2) presence of juvenile thalli (< 2 cm);
3) presence of meristematic lobes;
4) presence of lobes with vegetative propagules;
5) presence of fruiting bodies.

Data of each of the parameters from 2 to 5 are recorded adopting the following ordinal scales: i) absent; ii) sporadic: <10 thalli (or lobes or apothecia); iii) abundant: > 10 thalli (or lobes or apothecia).

5.2 Sampling equipment

Each crew will bring in the field appropriate standard forms, a tape measure, a hand lens, a ruler or a caliber, a camera.

5.3 Frequency of sampling

The assessment of *Lobaria pulmonaria* should be undertaken ideally at least every five years. In national assessments, repetitions of the surveys in shorter intervals (yearly assessment) may be useful to detect small changes in the sampled parameters.

5.4 Sample collection

The collection of specimens of *Lobaria pulmonaria* is not foreseen and strongly discouraged. On the other hand, the acquisition of photos of the specimens of *Lobaria pulmonaria* and/or of the features object of the survey is encouraged (e.g., presence of fruiting bodies, meristematic lobes, etc.).

5.5 Quality assurance and quality control

Training, intercalibration, and intercomparison courses should be organized at the national level. Data quality objectives and data quality limits should be defined on the basis of the results of the first intercalibration tests. In the meantime, it is possible to refer to the Data Quality Objectives and Limits set for lichen data collected by means of the ICP Field Manual part VII.2 (DQO: Control ± 20%; DQL: ≥80% of the teams fulfill the DQO).

6. Data handling

All data must be sent to the relevant National Focal Centre after each sampling year. Each Focal Centre is in charge of data validation.

6.1 Data processing

Each plot is classified according to its belonging to the class of abundance of *Lobaria pulmonaria* (rare, sporadic, abundant).

Based on the results obtained on the colonized trees, the following indicators are calculated:

- % trees belonging to each class of *Lobaria pulmonaria* abundance (rare, sporadic, abundant);
- % trees belonging to each class of abundance of juvenile thalli (absent, sporadic, abundant);
- % trees belonging to each class of abundance of meristematic lobes (absent, sporadic, abundant);
- % trees belonging to each class of abundance of vegetative propagules (absent, sporadic, abundant);
- % trees belonging to each class of abundance of fruiting bodies (absent, sporadic, abundant).

6.2 Data reporting

All validated data should be sent to the National Focal Centre and submitted annually to the transnational central data storage.

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4.6.3. LICHEN FUNCTIONAL DIVERSITY: GUIDELINES FOR THE ASSESSMENT OF EPIPHYTIC FRUTICOSE LICHEN DIVERSITY ON THE GROUND

1. Introduction

Fruticose lichens, and especially hair lichens, are particularly sensitive to pollution and climate change, as their large surface area to mass ratios filters moisture and elements from the air (e.g., Knops et al. 1996; Stanton et al. 2014), and they have strongly declined in areas with atmospheric pollution and intensive forestry (see Esseen et al. 2016). This makes this functional group a useful indicator of air pollution and climate change in forest ecosystems (see e.g., McCune 1994; Dettki and Esseen 2003; Lehmkuhl 2004; Nascimbene et al. 2019). Further, although their identification at the species level can be problematic, they are clearly visible, identifiable by the naked eye, in the field as a distinct morphological guild (Nascimbene et al. 2019).

This protocol, which is based on the assessment of the number and abundance of epiphytic fruticose lichen species on the ground (including litter, fallen twigs and branches), has been drafted starting from the recent literature in this field to be adopted as simplified method to the NEC Directive network.

2. Scope and application

This manual aims at providing a consistent methodology for the assessment and monitoring of epiphytic fruticose lichen diversity on the ground (including litter, fallen twigs and branches) of the sampling sites of the NEC Directive Network, as a new indicator of ecosystem integrity in forest plots.

3. Objectives

The main objective of this protocol is to assess, at the plot level, the occurrence of epiphytic fruticose lichens on the ground (number of species and their relative abundance) of the forest plots of the NEC Directive Network as an indicator of air pollution and climate change.

The study of the trends in this parameter in the NEC Network sites is expected to provide early warning responses to forest environmental changes, occurring in the medium- to long-term period.

4. Location of sampling

The assessment of the occurrence of epiphytic fruticose lichens on the ground is carried out at the plot level on Level II sites.

4.1 Sampling strategy

In each plot, 10 circular sub-plots (2 m radius) centered on 10 randomly selected trees (DBH ≥ 13 cm) are sampled for the collection of fruticose lichens on the ground. The random selection of the trees is carried out by means of the tree information available from the existing database at Level II (tree code), disregarding trees with DBH<13 cm.

5. Sampling

5.1 Data collection

In each subplot, the epiphytic fruticose lichen on the ground is collected.

5.2 Sampling equipment

Each crew will bring in the field appropriate standard forms, a tape measure, a hand lens, paper bags, a camera.

5.3 Frequency of sampling

The assessment of the occurrence and abundance of epiphytic fruticose lichens on the ground should be undertaken ideally at least every five years. In national assessments, repetitions of the surveys in shorter intervals (yearly assessment) may be useful to detect small changes in the sampled parameters.

5.4 Sample collection

All lichen thalli and fragments of pendulous lichens, including those on twigs and branches, are collected within each sub-plot and stored in paper bags, identified by the code of each subplot (e.g., CAL1-S1 to CAL1-S10).

5.5 Lichen species identification

This activity is carried out in the laboratory by means of microscope and with the use of identification keys. As far as possible, the collected specimens are identified at the species level.

5.5 Quality assurance and quality control

Training, intercalibration, and intercomparison courses should be organized at the national level. Data quality objectives and data quality limits should be defined on the basis of the results of the first intercalibration tests. In the meantime, it is possible to refer to the Data Quality Objectives and Limits set for lichen data collected by means of the ICP Field Manual part VII.2 (DQO: Control ± 20%; DQL: ≥80% of the teams fulfill the DQO).

6. Data handling

All data must be sent to the relevant National Focal Centre after each sampling year. Each Focal Centre is in charge of data validation.

6.1 Data processing

At the sub-plot level, the following indicators are considered:

- number of species of epiphytic fruticose lichens;
- relative abundance of each species. This is assigned adopting the following ordinal scale: i) rare: 1-3 thalli per sub-plot; ii) sporadic: 4-10 thalli per sub-plot; iii) abundant: > 10 thalli per sub-plot.

At the plot level, the following indicators are calculated:

- % of subplots with fruticose lichens;
- species richness descriptive statistics: mean number of species, min-max, standard deviation;
- total number of species;
- species abundance: % of rare, sporadic and abundant species at the plot level.

6.2 Data reporting

All validated data should be sent to the National Focal Centre and submitted annually to the transnational central data storage.

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4.7. COMMUNITY ANALYSIS: ACOUSTIC COMPLEXITY OF CHIROPTERA; RELATIVE ABUNDANCE (BASED ON VOCALISATIONS)

B2 macro category: Faunal diversity

Partner: CNR-IRET - Paolo Colangelo - Bruno De Cinti - Flavia Sicuriello

1. Introduction

Many bat species roost and/or forage in the forest or use forest patches and corridors for commuting and migration stopovers. The presence and activity of bats in the forest are highly influenced by the forest age and structure, and, in turn, forest management (Russo et al., 2021). Bats show responses to anthropogenic stressors linked to changes in other ecosystem components such as insects, and as K-selected mammals, can exhibit fast population declines. This speciose, widespread mammal group shows an impressive trophic diversity and provides key ecosystem services. For all these reasons, bats might act as suitable bioindicators in many environmental contexts (Jones et al., 2009; Jones et al., 2013). Despite the limited number of studies available in forest ecosystems (Cistrone et al., 2015; Carr et al., 2020; Piksa et al., 2022; Vlaschenko et al., 2022), the use of bats as bioindicators is highly promising (Tuneu-Corral et al., 2020) and warrants further investigation in specific contexts such as forestry and climate change (Russo et al., 2021).

The use of bats as bioindicators has pros and these comprise: a relative taxonomic stability; wide geographic ranges; rich trophic diversity; graded responses to environmental alteration correlated with those of other biodiversity components, such as insects; a rapid population declines due to slow population growth (Jones et al., 2009). More recently the use of Passive Acoustic Monitoring (PAM) for bat species detection allowed to increase data availability and in turn the possibility to test the use of bat assemblage as indicators of ecosystem health. Passive Acoustic Monitoring (PAM) does not require active presence of users for monitoring making it possible to standardize the collection of acoustic data (Froidevaux et al., 2014). However, although PAM allows for the collection of an unprecedented amount of data, attention must be paid to the difficulty of data analysis and validation by an expert is advisable (Russo and Voigt, 2016).

PAM of bat assemblages is still an emerging field in bat research and probably due to a general lack of methodological standards and the lack of common ecological indices, long-term bat acoustic monitoring programs are still scarce (Tuneu-Corral et al., 2020).

2. Scope and application

Presence of target bat species or functional groups community assemblages, species richness and evenness, activity levels can be affected by forest composition. PAM can be used to collect data on many different sites and test the possibility to use bats as bioindicators of forest health and composition. Despite the large amount of literature on the ecology of forest bats explicit examples of the use of bats as bioindicators in forest environments are scarce (Russo et al., 2021). Some indexes were proposed in literature (Tuneu-Corral et al., 2020) and their applicability to the NEC context should be evaluated.

3. Objective

The objective of this protocol is to suggest a possible monitoring sampling scheme to collect data on bat species using species-specific ultrasonic calls. The data will be used to calculate species richness and community composition. The obtained data will be used to evaluate the possibility of use species composition or presence and activity of selected target species

4. Location of measurements/assessments/sampling

The location corresponds to the 10 plots belonging to the *RETE NEC ITALIA*, the monitoring net, derived from the ICP Forests/CONECOFOR one. Recorders are the same used for Bird call detection and should be placed in the same tree used as origin for the eDNA transects (this potentially will allow a comparison of data). As for birds, it is important to collect data in the same period on all the sites.

5. Measurements/assessments/sampling (+QA|QC)

Measurements will be performed in accordance with the protocol proposed by LifeSpan project (https://www.lifespanproject.eu/), using the same acoustic devices used for birds that can be programmed by operators. Specifically, bats' calls will be recorded using a "song meter mini bat ultrasonic recorder" (Wildlife Acoustics). Little training is necessary to configure the device (using a user-friendly app) and to activate (the first day) and deactivate (the last day) the devices.

- Ultrasonic vocalization of bats will be recorded using the same devices used for birds. As for birds, the recorder will be set to be active for 5 days in late spring-early summer.
- Possibly avoid rainy days.
- Recorder must be tied to a tree (the same tree used as origin for the eDNA transects).
- Every night the recorder will be active from 1 hour before sunset to 1 hour before sunrise

6. Data handling

No data handling is required in the field except the GPS coordinates. The recorders are provided with an SD card. All the files will be downloaded and analyzed with dedicated software and validated by an expert.

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8. Annexes

4.8. COMMUNITY ANALYSIS: ACOUSTIC COMPLEXITY OF THE COMMUNITY AND TAXONOMIC DIVERSITY

B2 macro category: Faunal diversity

Partner: CNR-IRET - Paolo Colangelo - Bruno De Cinti - Flavia Sicuriello

1. Introduction

Forest bird species are appropriate indicators to test the effectiveness of ecological forestry because they occupy a broad range of forest habitat types and food sources, are responsive to the types of changes in forest conditions caused by forest management, vocally defend breeding territories, can be cost-effectively and unobtrusively monitored, and are a high conservation priority and responsibility for resource managers (Rempel et al. 2016). Thus, estimating avian biodiversity is a key measure of forest ecosystem condition.

Surveys conducted using Passive Acoustic Monitoring (PAM) by using autonomous recorder units (ARU), are efficient at estimating bird diversity (Metcalf et al., 2022; Darras et al., 2019). PAM does not require active presence of users for monitoring, making it possible to standardize the collection of acoustic data. When the focus is the study of the soundscape, which is defined as the product of the relationships between the sounds of the environment and the listener, it is important to standardize the time window for recording and recording should be done possibly simultaneously at different sites. Recording can then be used to calculate different acoustic indexes: Acoustic Complexity Index (ACI; Pieretti et al. 2011), Normalized Difference Soundscape Index (NDSI, http://www.real.msu.edu; Kasten et al. 2012), Bioacoustic Index (Boelman et al. 2007), Acoustic Diversity Index (ADI; Villanueva-Rivera et al. 2011), Acoustic Evenness Index (AEI, Villanueva-Rivera et al. 2011).

2. Scope and application

The scope of PAM will be the evaluation of differences in the soundscape between different forests and to test the efficiency of such monitoring to indicate forest status.

3. Objective

Bird calls will be recorded in a 2-hour window across sunrise. The obtained wave files will be used to calculate indexes of acoustic complexity (Shaw et al., 2021, Gasc et al., 2013) that will be correlated to forest health parameters. The results of many recent studies suggest the possibility of using PAM to monitor the bird population and the state of forest ecosystems (Atemasov and Atemasova 2019).

4. Location of measurements/assessments/sampling

The location corresponds to the 10 plots belonging to the *RETE NEC ITALIA*, the monitoring net, derived from the ICP Forests/CONECOFOR one

5. Measurements/assessments/sampling (+QA|QC)

Measurements will be performed using acoustic devices that can be programmed by operators. Specifically, birds' calls will be recorded using a "song meter mini bat ultrasonic recorder" (Wildlife Acoustics). Little training is necessary in order to configure the device (using a user-friendly app) and to activate (the first day) and deactivate (the last day) the devices.

- The recorder will be set to be active for 5 days between late spring and early summer. Possibly avoid rainy days.
- Recorder must be tied to a tree.
- Every day the recorder will be active from 1 hour before sunrise to 1 hour after sunrise and automatically collect data.

6. Data handling

No data handling in the field is required except recording the number of the device and the GPS coordinates. The recorders are provided with an SD card. All the files will be downloaded and analyzed with dedicated software.

7. References

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8. Annexes

4.9. ENVIRONMENTAL DNA (eDNA): TARGET TAXA INVERTEBRATES AND MAMMALS

B2 macro category: Faunal diversity

Partner: CNR-IRET - Paolo Colangelo - Bruno De Cinti - Flavia Sicuriello

1. Introduction

Terrestrial animal community monitoring has traditionally relied on the physical identification of species by trapping, visual surveys, and individual enumeration. However, traditional monitoring techniques may remain problematic due to difficulties associated with the identification and shyness of individuals, adverse environmental conditions, etc. In this context, recent advances in molecular techniques offer new opportunities for monitoring the terrestrial ecosystem (Bohmann et al., 2014). Methods based on environmental DNA (eDNA) now allow to produce inventories of indicator taxa which can subsequently be used to assess biodiversity and ecological quality (Thomsen et la., 2015, Deiner et al., 2016). However, integrating these new DNA-based methods into current monitoring practices is not straightforward and will require additional efforts for testing field and lab protocols (van der Heyde et al., 2022). Technologies related to eDNA were initially developed for microbes, but eDNA is now routinely used for plants and large animals in all types of ecosystems. In recent years, much research has focused on developing workflows to assess the diversity of terrestrial animal communities. With regard to mammals, some pivotal studies have been conducted to evaluate the reliability of eDNA detection and species identification. These works have been tested both in a controlled environment (such as a zoo) and in a wild environment leading to interesting and promising results (Andersen K et al., 2012; Rodgers TW & Mock KE, 2015; Ushio M et al., 2017; Leempoel et al., 2020).

2. Scope and application

The presence and absence of different taxa (mammals and arthropods) will be detected and used to calculate species richness and community composition. It is known that forest composition, health status, fragmentation etc. affect animal species richness and functional diversity. eDNA was proved to be sensitive to species presence and can help to integrate animal diversity into long-term forest ecosystem monitoring.

3. Objective

The proposed protocol is aimed at suggesting a sampling scheme to assess and monitor animal species assemblages by using genetic techniques. We will focus on specific taxonomic groups such as small mammals that are recognized as a valuable group of wildlife for understanding fine-scale responses to environmental change (Avenant 2000, Balčiauskas et al., 2017). Small mammals are intermediate trophic components of communities where they rely on vegetation, seeds, and arthropods for food, and interact with higher vertebrates through competition for resources or as a source of prey (Krebs et al. 2014). Moreover, we will explore the invertebrate soil community diversity and its correlation with more standard monitoring methods such as QBS-ar.

4. Location of measurements/assessments/sampling

Soil samples will be collected once a year in the 10 plots belonging to the *RETE NEC ITALIA*, the monitoring net, derived from the ICP Forests/CONECOFOR one Seasonality and environmental conditions (temperature) can affect communities' composition and eDNA detection success. Thus, it is important to collect soil samples in the same time window, preferably during late spring-early summer in concomitance with bats and birds acoustic monitoring. Late summer should be avoided because of too high temperatures and dryness.

5. Measurements/assessments/sampling (+QA|QC)

Collecting soil for eDNA is not a difficult task and does not require specific training. It is important to work wearing gloves and using clean spoons to avoid cross contamination of replicates. It is also important to transfer soil samples as soon as possible to the final destination for DNA extraction. Alternatively, the samples should be stored at -20°C.

1. Starting from a fixed point (corresponding to the tree where the bats and birds recorder will be placed), take soil samples along four 5 meter transects (in the direction of the four cardinal points).
2. Walk 5 meters from the origin (the tree) in one direction collecting approximately 10 mL of soil each meter and putting soil in a plastic bag (provided). Before taking up the soil, clean the ground surface of leaves and other debris.
3. Repeat step 2 along the other three directions. Use a clean spoon to take up the soil (one spoon for each transect). Always wear gloves. Falcons, spoons, gloves and measuring tape are supplied with the kit.
4. Store the samples at -20°C as soon as possible. If that is not possible, put it in the refrigerator at 4°C.

6. Data handling

No data handling is required in the field. Soil samples will be processed in the laboratory in order to extract DNA using a specific kit. Successively a PCR amplification of mitochondrial 12S gene (or cytochrome oxidase I) will be performed. Obtained amplicons will be sent to external services for sequencing. Obtained *fastq* files will be processed using specific bioinformatic pipelines in order to identify taxa

7. References

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8. Annexes

4.10. COMMUNITY ANALYSIS: TAXONOMIC DIVERSITY– SOIL BIOLOGIC QUALITY OF ARTHROPODS (QBS-AR)

B2 macro category: Faunal diversity

Partner: CNR-IRET - Paolo Colangelo - Bruno De Cinti - Flavia Sicuriello

1. Introduction

QBS-ar was developed in recent years by an Italian team (Parisi, 2001; Parisi et al., 2005). The term "QBS" is the acronym of Soil Biological Quality (in Italian: Qualità Biologica del Suolo) and "ar" refers to the arthropod community. The index is based on soil invertebrates that belong to the phylum of arthropods with a size between 0.2 and 2 mm (mesofauna).

Edaphic microarthropod communities are an important reservoir of biodiversity and play an essential role in several soil ecosystem functions; furthermore, they are often used as soil quality indicators (Menta et al., 2011).

In forest ecosystems, soil and litter arthropod communities control the decomposition of fresh organic matter and the formation of the humus profile (Ponge et al., 1986; Bird et al., 2000). Soil fauna affects litter decomposition by several mechanisms: fragmentation of leaf litter, biochemical modification of organic matter during the passage through the intestines, mixing of organic and mineral particles, increase of microbial activity, destruction and dissemination of fungal hyphae and spores (Hedde et al., 2007; Coulis et al., 2013).

QBS-ar index focuses on the presence of morphological characters that indicate the degree of adaptation to soil such as the reduction or loss of pigmentation, streamlined body form, reduced or loss of appendages (hairs, antennae, legs), reduction or loss of structure to fly, jump or run, reduced visual apparatus, reduced water-retention capacity and presence of specialized organs (i.e., post-antennal organs). Soil community is therefore composed by epi-edaphic, hemi-edaphic and eu-edaphic taxa progressively more closely related to the hypogeal environment and intolerant towards the disturbance.

The index is based on the concept that the higher is the number of microarthropod groups morphologically well adapted to this soil habitat and the higher is the soil quality (Joseph et al., 2016). So, this index combines the presence of microarthropods in the soil, intended as biodiversity, and their capability to adapt to soil conditions intended as vulnerability. (Menta et al., 2018)

2. Scope and application

The primary scope of QBS-ar index is to measure the degree of adaptation to the hypogeal life of microarthropods and, on the application side, to provide a numerical value resulting from a synthetic index of biological quality of a soil. From the operating point of view this index has the objective of overcoming the difficulties of taxonomic analysis at the species level and can also be used by non-specialists following short training. A point of strength of this protocol is to be inexpensive, in terms of equipment, time and energy for sampling and analyzing samples.

Among several indices developed to evaluate soil biodiversity and soil biological quality, QBS-ar is having a good diffusion around Europe: it has been used successfully to test for the effects of forest cutting, grazing, trampling, industrial activities, emission, agriculture, heavy metals, and other anthropogenic effects (Romeo et al., 2020; Gardi, Menta, & Leoni, 2008; Menta et al., 2014); it has been applied to several ecosystems, including agricultural lands, grasslands, urban soils, woods at different level of wilderness, and degraded soils (Menta et al., 2018).

Because of these characteristics QBS-ar index was considered to be a standard protocol for measuring soil fauna across Europe LTER sites ExpeEr Ecosystem Research Program (Experimentation in Ecosystem Research, proj. no. 262060, (http://www.expeeronline.eu/about-expeer/context.html, Firbank et al., 2017), and it is reported in the European Commission DG ENV, 2010. In addition, the index was inserted in the COMMON GUIDELINES FOR ANALYTICAL METHODS (Malusà et al., 2019; D'Avino et al., 2022)

3. Objective

The high number of applications in Italy, European and non-European countries signal the potential of QBS-ar. It is a good candidate index for continuous biomonitoring of soil communities to describe patterns and processes in the microarthropod biodiversity across the landscape. A deeper knowledge of soil biodiversity in response to landscape use will provide guidance in effective management planning for sustainable renewable resource use and nature conservation. (Joseph et al., 2016)

4. Location of measurements/assessments/sampling

Under stable conditions such as woods, soil can be sampled once a year, in the same period, preferably spring or autumn. Winter and summer are to be discarded because of too low temperature conditions and dryness that can reduce the presence and activity of soil fauna. Samples for QBS-ar calculation have to be collected when soil moisture ranges between 40% and 80% of field capacity (Parisi et al., 2005).

5. Measurements/assessments/sampling (+QA|QC)

The respect of the protocol for the extraction stage is particularly important for the quality assurance of the indicator. The operation requires trained personnel for the four steps of the protocol: soil sample, animal extraction, identification of groups and scoring. By focusing on the presence of characteristics of adaptation to soil environments, and not requiring the complex taxonomic identification to the species level, QBS-ar analysis can also be performed by non-specialists (Galli et al., 2014) after a few days training.

Protocol of QBS-ar follows four phases (Parisi et al., 2005, Menta et al.,2008; Joseph et al., 2016; D'Avino et al., 2022):

1. Soil sampling - An appropriate sampling area 10X10m should be chosen in the study site, taking into account the high variability of the soil communities and the possible margin effect. For each sampling unit, three cubic soil samples of 1 dm^3 (10x10x10cm) are taken inside the sampling area after removing the litter layer. Samples can be stored in plastic bags away from thermal shock and impact and must be placed in the extraction set-up as soon as possible and in any case within 48 hours.

2. Microarthropod extraction – Microarthropods are extracted from soil cores using a Berlese-Tullgren (Berlese, 1905; Tullgren, 1918; Southwood, 1994) funnel in which heat from a lamp causes the arthropods to escape. Funnel consists of an incandescent lamp (40–60 Watt) placed 30 cm up the soil sample, a sieve (mesh of 2 mm, 20 cm in diameter), a funnel (plastic or glass), a container with a fixer liquid (2/3 alcohol and 1/3 glycerol). The duration of microarthropod extraction from soil is in relation to the soil moisture (never less than 5 days). The respect of the protocol for the extraction stage is particularly important for the quality assurance of the indicator.

3. Determination of biological forms and assignment of the Ecological-Morphological Index (EMI) – The extracted specimens are observed using a stereomicroscope at low magnification (40X) and classified at order/class level.

   Within each higher taxon, QBS method requires searching for the biological form (morpho-type) that is most adapted to soil. This type will receive an eco-morphological score (EMI), proportionate to its adaptation level (see Annex). As a general rule, eu-edaphic (i.e., deep) forms get an EMI = 20, hemi-edaphic forms (i.e., intermediate) are given an index rating proportionate to their degree of specialization, while epi-edaphic (surface-living) forms score EMI = 1.

   The microarthropods are identified by class for myriapods (Diplopoda, Chilopoda, Symphyla, Pauropoda) and order for insects, Chelicerata and Crustacea. The specimens belonging to each taxon are then counted and separated into biological forms. Each form is associated with a score (EMI—Eco- Morphological Index), which ranges from 1 to 20 in proportion to its degree of adaptation to soil.

4. QBS-ar index computation – It results by sum of EMI values obtained in the extracted sample. Whenever two EMI values are assigned at the same taxon, it must be considered the higher EMI value for the QBS-ar computation.

5.

6. Data handling

Field data entry will be done using a paper form, then the survey teams will transcript to an electronic sheet each field form. Specific software packages will be used to compute indices, with a preference for open-source systems (e.g., R).

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8. Annexes

Biological forms identification with EMI score (modified from D'Avino et al., 2022)

Subph / class	Biological Form (BF)_ Ecomorphological index (EMI)	
Arachnida	semi-mobile gnathosoma, forelegs forward	***Acarina_20***
	spider ≥ 5mm: fangs chelicerae, ceph.+abdomen	***Araneae_1***
	spider <5mm & scarcely pigmented	***Araneae_5***
	as *scorpiones* without abdomen extension	***Pseudoscorp._20***
	eyes, 2 body segments fusion, comm. long legs	***Opiliones_10***
	big pedipalps with pincers (*juv.* forms)	***Scorpiones_10***
	locomotion palps and whip-like flagellum	***Palpigradi_20***
Crust.	7 pairs of jointed limbs 1 long pair of antennae	***Isopoda_10***
Myriapoda	slender antennae, 12 pair fo legs (in adult), cerci	***Symphyla_20***
	cilindrical body, 2 pairs of legs per segment	***Diplopoda_10***
	Diplopoda < 5mm or *Polixenida*	***Diplopoda_20***
	flattened body, 1 pair of legs (>15) per segment	***Chilopoda_10***
	Chilopoda < 5mm or *Geophilomorpha*	***Chilopoda_20***
	small, pale, branching antennae, 9-11 pair of legs	***Pauropoda_20***
Entognatha	spr.>2mm, complex pigment.,developed append.	***Collembola_1***
	pigmented, well developed append. and eyes	***Collembola_2***
	small size, scarce pigment., medium append.	***Collembola_4***
	modest pigm., small append., developed eyes	***Collembola_6***
	modest pigm., very small append., short/no furca	***Collembola_8***
	very small size, no pigmentation, small furca	***Collembola_10***
	no pigmentation, no furca, short appendages	***Collembola_20***
	no eyes, long and slender abdomen with 2 cerci	***Diplura_20***
	conical head, no eyes or anten., first legs forward	***Protura_20***
Insecta	elytra and protorax big and mobile: epigeic forms	***Coleoptera_1***
	+5 EMI points each character: dimension <2mm; depigmentation (tan/brown); reduction/absence of eyes; reduction/absence of wings	***Coleoptera_5***
		Coleoptera_10
		Coleoptera_15
		Coleoptera_20
	3 pairs of legs, comm. sclerotized head capsule	***L-Coleoptera_10***
	2 wings and 2 halteres	***Diptera_1***
	Larva, mostly no legs, small or absent head	***L-Diptera_10***
	4 wings, antennae longer than heads	***Hymenoptera_1***
	Formicidae, globose abdomen	***Hymenoptera_5***
	Larva, mostly no legs	***L-Hymenoptera_10***
	piercing-sucking mouthparts	***Hemiptera_1***
	Cicada nymph	***N-Hemiptera_10***
	hypognate mouth apparatus, long antennae	***Psocoptera_1***
	fringed wings, abdomen with 11 segments	***Thysanoptera_1***
	elonged abdomen with 10 segments	***Embioptera_10***
	a pair of cerci above their posterior	***Dermaptera_1***
	cerci, reduced wings, hypognate mouth	***Blattaria_5***
	elonged abdomen with 11 segments	***Archaeognatha_10***
	Larva bottle shape, comm. with prolegs	***L-Lepidoptera_10***
	no wings, abdomen with short cerci	***Isoptera_10***
	sylvery scales, abdomen with styles in urites	***Zygentoma_10***
	a pair of fan wings, elonged hindlegs	***Orthoptera_1***
	mole cricket *juveniles*	***Orthoptera_20***
	strongly elongated rostrum, scorpionflies	***Mecoptera_1***
	Larva scarabaeiform or eruciform (caterpillar-like)	***L-Mecoptera_10***
	large lateral compound eyes, net-wings	***Neuroptera_1***
	Larva slender campodeoid, postcephalic sclerites	***L-Neuroptera_10***
	prothorax elongated, bilobed 3rd tarsomere	***Raphidioptera_1***
	Larva campodeiform, proth.elong., unsclerotized abd.	***L-Raphidioptera_10***

4.11. POLLUTANT RETENTION CAPABILITY OF THE SOIL-FOREST SYSTEM

B2 macro category: Pollutant fluxes

Partner: UNIFI-DST - Stefano Carnicelli and Anna Andretta

1. Introduction

This chapter describes the procedures which will be tested, within Action B.3, to estimate the fluxes of water, from precipitation to soil drainage, occurring in the selected monitoring sites, namely the four new sites being established in Action B.1 and two existing sites which have been included as reference, thanks to their long and continuous data series. This new method application is intended to improve the simple flux estimate obtained within a previous LIFE project, LIFE SMART4Action, LIFE13 ENV/IT/000813 (Cecchini et al, 2019, 2021). These estimates, obtained from soil solution monitoring, revealed how, in sites with high nitrogen depositional load, the issue of release of active N from the soil to underground waters may actually be serious. It is then necessary to obtain more accurate estimates of water fluxes, in order to quantify the magnitude of polluting N flows caused by atmospheric deposition. When developed, the method will be usable for other pollutants, too.

2. Location of measurements/assessments/sampling

Samplers for precipitation and throughfall volume, sensors for meteorological parameters and for soil water content must be placed according to the ICP-Forests manual, part IX Meteorology,

http://icp-forests.net/page/icp-forests-manual

3. Measurements/assessments/sampling (+QA|QC)

Measurements of precipitation and throughfall volume, meteorological parameters and soil water content must follow the ICP-Forests manual, part IX Meteorology,

http://icp-forests.net/page/icp-forests-manual

including the relevant procedures for QA/QC.

4. Data handling

Estimation of water fluxes through and out of the soil requires implementation of a soil water mathematical model. According to the scale and spatial parameters implicit in the measurement site philosophy, and to the kind of questions that such a monitoring frame can answer, a 1-dimensional (1D) soil water model is considered to be adequate. It is expected that the most critical step in implementing this kind of models on NEC monitoring sites will be the estimation of evapotranspiration (ET).

Examination of available soil water models has evidenced that few such models exist which are specifically conceived for application in a forest context. A recent specialized model is SWUF (Paul et al, 2003). This model is a modern software concept and simple to use; however, it has been tested, validated and parametrized in Australia only, and thus offers limited guarantees of good estimations in our, quite different, environment.

One classical model is BROOK90 (Hammel & Kennel, 2001; Federer, 2002), which has been specifically developed for use in forest environments. The model in itself is relatively old, but continuous updates and adaptations to modern software standards make it still quite interesting. One potential issue with this method is that it allows estimation of ET exclusively through the Penman-Monteith model. Application of this last model would require a large volume and diversity of input data, not all of which are available. Existing, published, applications in European contexts (Wegehenkel et al, 2017; Schmidt-Walther et al, 2020, Meusburger et al, 2022) suggest that these problems can be solved. The SWAP model (Kroes et al, 2008) was mainly designed for use in agricultural environments, but successful applications in forest environments have been published (Wessolek et al, 2008; Rosenqvist et al, 2010a, b; Liu et al, 2012). The model is highly engineered and offers many alternatives, so that it can be adapted to simulate soil-forest systems; an important consideration is that this model offers alternative options for the estimation of evapotranspiration, which require less input data and might actually result in better approximations. From the considerations above, it has been initially decided to practically test, within action B.3, both BROOK90 and SWAP models, profiting from the fact that preparation of input data ls largely the same for both models. Comparative tests will offer more insight on the performance of the models.

Input meteorological data will be taken from the normal monitoring data for the sites; soil hydraulic parameters will be estimated using the ROSETTA software (Schaap et al, 2001, 2204; Zhang and Schaap, 2017) using existing or in progress soil analytical data, including bulk density. Model results will be validated and calibrated using TDR soil water content measurements, taken at the same depths of soil water samplers, MODIS remote sensing evapotranspiration data for past years and the results obtained by using Cl as tracer (Cecchini et al, 2019, 2021).

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