

Guidelines for the use of lichens as bioaccumulators





ISPRA
Istituto Superiore per la Protezione
e la Ricerca Ambientale



Sistema Nazionale
per la Protezione
dell'Ambiente



Società
Lichenologica
Italiana

Guidelines for the use of lichens as bioaccumulators

Legal Disclaimer

The Institute for Environmental Protection and Research (ISPRA), together with the 21 Regional Agencies (ARPA) and Provincial Agencies (APPA) for the protection of the environment, as of 14 January 2017 is part of the National Network System for the Protection of the Environment (SNPA), established by the Law June 28, 2016, n.132.

The Institute for Environmental Protection and Research, or persons acting on its behalf, are not responsible for the use that may be made of the information contained in this report.

ISPRA - Istituto Superiore per la Protezione e la Ricerca Ambientale

Via Vitaliano Brancati, 48 – 00144 Roma

www.isprambiente.gov.it

ISPRA, Manuali e Linee Guida 189bis/2019

ISBN 978-88-448-0967-6

Extracts from this document may be reproduced on the condition that the source is acknowledged

Graphic design

Cover design: Sonia Poponessi

ISPRA – Communications Area

Cover photo: Silvana Munzi

Italian Lichen Society (*S.L.I.*)

Coordination of the online publication:

Daria Mazzella

ISPRA – Communications Area

January 2020

Authors

This Guideline was conceived and developed by the Working Group on Biomonitoring of the Italian Lichenological Society (SLI).

Paolo Giordani (Università di Genova, Working Group coordinator)

Renato Benesperi (Università di Firenze)

Elisabetta Bianchi (Università di Firenze)

Giorgio Brunialti (TerraData environmetrics)

Elva Cecconi (Università di Trieste)

Tania Contardo (Università di Siena)

Luca Di Nuzzo (Università di Firenze)

Lorenzo Fortuna (Università di Trieste)

Luisa Frati (TerraData environmetrics)

Stefano Loppi (Università di Siena)

Fabrizio Monaci (Università di Siena)

Silvana Munzi (Universidade de Lisboa)

Juri Nascimbene (Alma Mater Università di Bologna)

Luca Paoli (Università di Pisa)

Sonia Ravera (Università di Palermo)

Mauro Tretiach (Università di Trieste)

Andrea Vannini (Università di Siena)

CONTENTS

1. INTRODUCTION	7
1.1 Environmental biomonitoring.....	7
1.2 Biomonitoring and the Italian legislation	7
1.3 Lichens as biomonitors of airborne elements	8
1.4 Principles	8
2. COMMON GROUNDS BETWEEN THE METHOD OF NATIVE LICHENS AND THE METHOD OF LICHEN TRANSPLANTS	9
2.1 Scope and goal.....	9
2.1.1 <i>Applicability conditions</i>	9
2.1.2 <i>Study objective</i>	9
2.1.3 <i>Sampling objective</i>	9
2.2 Terms and definition.....	9
2.3 Equipment.....	11
2.3.1 <i>Field work preparation equipment</i>	11
2.3.2 <i>Field equipment</i>	11
2.3.3 <i>Laboratory equipment</i>	11
3. GUIDELINES FOR BIOACCUMULATION OF ELEMENTS USING NATIVE LICHENS	13
3.1 Characterization of the study area	13
3.2 Sampling design	13
3.2.1 <i>Sampling unit (SU)</i>	13
3.2.2 <i>Sampling density</i>	13
3.2.3 <i>Suitable lichen species</i>	14
3.3 Fieldwork.....	14
3.3.1 <i>Sample collection</i>	14
3.4 Preparation of analytical samples	15
3.4.1 <i>Selection of suitable lichen material</i>	16
3.4.2 <i>Sample grinding and homogenization</i>	16
3.4.3 <i>Dry weight determination</i>	16
3.4.4 <i>Sample storage</i>	16
3.5 Analytical procedures	16
3.5.1 <i>Chemical analysis</i>	17
3.5.2 <i>Digestion method</i>	17
3.6 Expression of results.....	17
3.7 Interpretation of results.....	18
3.7.1 <i>Basic statistical analyses of biomonitoring data</i>	18
3.7.2 <i>Interpretative tool: the bioaccumulation scale for native lichens</i>	18
3.7.3 <i>Attribution of the bioaccumulation class to the Sampling Unit</i>	19
4. GUIDELINES FOR BIOACCUMULATION OF ELEMENTS USING LICHEN TRANSPLANTS	22

4.1	Lichen exposure in the study area: planning	22
4.1.1	<i>Characterization of the study area</i>	22
4.1.2	<i>Sampling design</i>	22
4.1.3	<i>Recommendations for sample exposure in the SUs</i>	22
4.1.4	<i>Estimate of the sampling effort in the background area</i>	23
4.1.5	<i>Exposure duration</i>	23
4.1.6	<i>Ancillary information</i>	23
4.2	Collection of the lichen material	23
4.2.1	<i>Suitable lichen species</i>	23
4.2.2	<i>Characterization of the background area</i>	23
4.2.3	<i>Procedures for lichen collection</i>	24
4.2.4	<i>Morphometric characterisation of the lichen material</i>	24
4.3	Laboratory procedures	24
4.3.1	<i>Preparation of bulk lichen material</i>	24
4.3.2	<i>Sample preparation</i>	25
4.4	Installation and retrieving of samples.....	25
4.5	Laboratory preparation of analytical samples.....	25
4.5.1	<i>Selection of suitable lichen material</i>	26
4.5.2	<i>Sample grinding and homogenization</i>	26
4.5.3	<i>Dry weight determination</i>	26
4.5.4	<i>Sample storage</i>	26
4.6	Analytical procedures	27
4.6.1	<i>Chemical analyses</i>	27
4.6.2	<i>Digestion method</i>	27
4.7	Expression of results.....	27
4.8	Interpretation of results.....	27
4.8.1	<i>Basic statistical analyses of biomonitoring data</i>	27
4.8.2	<i>Interpretative tool: the bioaccumulation scale for lichen transplants</i>	28
4.8.3	<i>Attribution of the bioaccumulation class to the Sampling Unit</i>	28
5.	RECOMMENDATIONS FOR QUALITY ASSURANCE AND QUALITY CONTROL ...	31
	ANNEX A. Workflow of standard main phases	33
	ANNEX B. Hints for the identification of suitable lichen species	35
	ANNEX C. Examples of transplants and exposure devices^T	43
	ANNEX D. Example of field sheets	44
	ANNEX E. Information needed at the end of the biomonitoring survey	47
	ANNEX F. Probabilistic sampling methods	48
	ANNEX G. Preliminary assessment of the lichen elemental content variability in a potential background area^T	51
	ANNEX H. Mapping procedures	56
	REFERENCES	58

1. INTRODUCTION

1.1 Environmental biomonitoring

Environmental monitoring through living organisms (biomonitoring; see definition in sect. 2.2) can be very effective to detect environmental changes. This approach is based on the assumption that any change taking place in the environment has a significant effect on the biota, and thus the key feature for using biological indicators to monitor the state of the environment is that they must clearly reflect any change in the environment itself (Keddy, 1991). Under these circumstances, biomonitoring is an effective method for investigating the impact of external factors on ecosystems. Among these, air pollution plays a major role since its negative impact on the environment and human health is definitely recognized.

Biomonitoring of air pollution has a long history, dating back to the second half of the 19th century, when the Finnish botanist William observed that epiphytic (tree-inhabiting) lichens in Paris were severely damaged by air pollution and could be used as bioindicators of air quality. It is important to remark that the output of biomonitoring studies is different from that obtained through diffusion modeling or active and passive physico-chemical devices: while the latter refer to emission data or ambient concentrations of pollutants, biomonitoring evaluates the biological effects of pollutants (Markert et al., 2003). Noteworthy, although the use of biomonitors is usually recommended as complementary rather than alternative to conventional monitoring by instrumental devices or analysis of bulk depositions, measurement of ambient concentrations of pollutants is of minimal use to evaluate their biological impact (e.g., the economic damage caused by a reduced performance of agricultural plants exposed to pollutants can hardly be predicted by any physico-chemical device; Loppi, 2014).

1.2 Biomonitoring and the Italian legislation

Directive 2008/99/EC of the European Parliament and of the Council sets measures for the protection of the environment through criminal law. In Italy this Directive finds its first application with the Legislative Decree 121/2011 dealing with the protection of endangered or threatened animal and plant species, as well as some important habitats. However, it is only with the Law 68/2015 that the Italian legislation introduced into the penal code a part (title VI bis) specifically devoted to crimes against the environment, consisting of twelve articles, among which five new articles dedicated to the protection of the environment and the ecosystem. In this framework, it is especially relevant the article 452 bis entitled "environmental pollution": with this article the legislator specified that are punishable those who cause significant and measurable impairment or deterioration of (i) water, air or soil, (ii) ecosystem, biodiversity, flora or fauna. Thus, with Law 68/2015 the environment as such acquired for the first time in Italy the merit of being protected, while before 2015 the environment was only defended in order to preserve human safety and health.

In Italy biomonitoring techniques have been widely applied to determine air, soil and water quality, as testified by a huge body of literature. Nonetheless, only biomonitoring of freshwater quality became enforced by law (at first with the Legislative Decree 152/1999, later replaced by the Legislative Decree 152/2006, putting into effect Directive 2000/60/EC that had established a general framework in the field of water policy). As far as air quality is concerned, Italy put into effect Directive 2008/50/EC on ambient air quality and cleaner air for Europe with the Legislative Decree 155/2010, which, for the evaluation of air quality, specifically refers to the use of bioindicators for the assessment of the effects on ecosystems caused by arsenic, cadmium, nickel, mercury and polycyclic aromatic hydrocarbons (art 5, sect. 12). Unfortunately, after nine years the applicative measures of such Decree are still missing.

Environmental monitoring is fundamental for Environmental Impact Assessment (EIA) since it allows to check the impacts of any work/action on the environment through monitoring of physical, chemical and biological parameters. The main objective of any EIA is the characterization of the baseline environmental conditions before the work/action ("ante operam") and the evaluation of changes after the work/action has been executed and put into operation ("post operam"). To achieve this goal, an Environmental Monitoring Plan (EMP) is required, which must specify the parameters/indicators to be investigated, the methodological approach, the number and location of sampling sites (MATTM, 2014). In detail, for any parameter/indicator, the EMP shall specify the Regulatory Acceptable Value (RAV), which, in turn, determines whether a predicted or measured environmental value is acceptable or not. Missing a RAV, criteria and methods for the definition of an environmentally relevant value (ERV) must be detailed since these may greatly affect the results; in addition, to avoid giving the false impression that results are based on a well-defined threshold value, the range of "natural" variability of the ERV has to be defined (MATTM, 2014). This latter is the case of biological monitoring of air quality.

1.3 Lichens as biomonitors of airborne elements

Biological monitoring proved to be very useful in the assessment of trace element air pollution (Manning & Feder, 1980): in particular, the use of lichens for assessing atmospheric levels and deposition patterns of trace elements is well-established (Bačkor & Loppi, 2009). Attempts to correlate elements accumulated by lichens with atmospheric concentrations suggested that lichens mainly reflect bulk (wet and dry) depositions (Pilegaard, 1979; Sloof, 1995; Godihno et al., 2008; Loppi & Paoli, 2015), but some studies indicated that lichens preferentially accumulate elements from particulate pollution (Glenn et al., 1991; Bari et al., 2001; Costa et al., 2002). As a consequence, lichens are becoming recognized as an important tool in environmental forensics (Tretiach et al., 2011; Purvis et al., 2013; Contardo et al., 2019).

Recently, the use of lichen transplants has been generally preferred over the use of native lichens (see definitions in sect. 2.2) for several reasons. First of all, the sampling design can be set up and realized properly without the constraints of finding suitable lichen-carrying trees and above all, of finding native lichen samples, possibly of the same species at all monitoring sites, in heavily polluted areas, where lichens can be very rare or even absent (“lichen desert”). Secondly, the lichen exposure time span is known and there is no need to use estimates of annual growth (Fortuna & Tretiach, 2018). Thirdly, the interpretation of results can be based on the knowledge of the concentration values measured in exposed and unexposed samples (sect. 2.2). Expressing the results as Exposed-to-Unexposed (*EU*) ratios allows for spatial and temporal comparisons, regardless of differences in the initial concentrations of unexposed samples (Fрати et al., 2005; Cecconi et al., 2019a, Loppi et al., 2019). In addition, it allows to investigate the accumulation capacity of different species under the same conditions (Bergamaschi et al., 2007). The limit of this method is that it cannot be used in case of unforeseen surveys, for example in case of a major accident to an industrial plant. In this latter case the use of native lichens may turn very useful to fulfil the requirements of the so-called Seveso Directives (82/501/EC, 96/82/EC, 2012/18/EU).

Despite the huge literature on the use of native and transplanted lichens as bioaccumulators of trace and major elements, there is a bewildering array of procedures available for almost all the steps involved, from the sampling strategy to sample processing, chemical analysis, data interpretation, and quality control. Such a lack of consistency often hampers any possibility of comparing different studies and greatly affects data quality. This document contains guidelines and practical hints to fulfil each phase of an epiphytic lichen-based survey targeting trace and major elements, with the main aim of providing two shared standard methods for bioaccumulation studies relying, respectively, on native and transplanted lichens. A special emphasis is given to the crucial point of the interpretation of results, which has notoriously important outcomes for decision making and environmental forensics. This issue, extensively addressed in the context of human biomonitoring and chemical risk assessment (e.g., Hays et al., 2007; Clewell et al., 2008) has also been faced in the field of lichen biomonitoring, with the development of different interpretative tools for native and transplanted lichens. Here, through the implementation of basic ideas underlying previous interpretative scales (Nimis & Bargagli, 1999; Frati et al. 2005), new dimension-less, species-independent “bioaccumulation scales” for native and transplanted lichens are adopted, which overcome most critical points affecting the previous scales, since they are based on the concept that pollution can be quantified by dimensionless ratios between experimental and benchmark values (Cecconi et al., 2019a).

1.4 Principles

The procedures described in these guidelines are applicable for the purposes of collecting bioaccumulation data of macro- and trace elements in lichens by means of two methods: the method of native lichens and that of lichen transplants. In order to guarantee data quality, the biomonitoring surveys applying these guidelines must be conducted by personnel and institutions with the necessary level of expertise in recognizing suitable biomonitor species (Annex B), processing biological material for analytical determination, and planning probabilistic sampling design (sect. 2.2, Annex F).

2. COMMON GROUNDS BETWEEN THE METHOD OF NATIVE LICHENS AND THE METHOD OF LICHEN TRANSPLANTS

The following sections describe aspects common to the two methods. Sections are labelled by the superscript *N* or *T*, respectively when contents refer to materials and methodologies suitable for either native lichens or lichen transplants.

2.1 Scope and goal

These guidelines are based on the review of best practices and results of recent methodological and applicative studies. The guidelines address the use of epiphytic lichens (bioaccumulation by native lichens and lichen transplants) to monitor spatial and temporal trends of element concentrations (macro-elements and trace elements) related to atmospheric pollutant depositions. In this document, all the phases of a biomonitoring survey are separately addressed. Moreover, a guidance framework and practical details concerning the equipment and protocols for the collection, preparation, exposure and retrieving of lichen samples are made available, along with good practices for the elaboration, expression, and interpretation of results.

Note. As far as lichen transplants are concerned, these guidelines do not include indications on the so-called ‘lichen bags’ technique (i.e., transplant-based studies in which selected portions of lichen material are air-exposed in nylon net bags; Adamo et al., 2003), since it has originally set up for mosses, and because the exposure of entire lichen thalli can be more easily performed (sect. 4.3.2).

1. Applicability conditions

The native lichens method is applicable in areas where the suitable lichen species occur with a homogeneous spatial distribution and in a sufficient amount in each sampling unit (sect. 2.2, cfr. Chapter 3).

The transplant method is applicable in the same conditions of the native lichens method, but also in areas (i) without native lichens (e.g. within the so-called “lichen desert”), (ii) with an irregular distribution of suitable species, or (iii) with insufficient amount of samples for chemical analysis (cfr. Chapter 4).

2. Study objective

Studies can be classified, with respect to their time span, in:

- i. Biomonitoring studies aimed at quantifying for the first time the concentrations of major and trace elements in lichen thalli (baseline studies) within a study area (sect. 2.2).
- ii. Biomonitoring programs based on a series of repeated measurements in the same study area over time. In this case, the objective is the detection of changes in concentration of macro- and trace elements over time. Biomonitoring surveys carried out to compare the effects of pollutant emissions referring to ante- and post-operam scenarios are an example of such an approach.

3. Sampling objective

For the native lichen method, the sampling objective is to obtain a measure of the concentrations of macro-elements and trace elements in samples of native lichens collected in the study area.

For the lichen transplant method, the sampling objective is to assess the magnitude of the enrichment of macro-elements and trace elements in lichen samples transplanted in the study area (i.e. concentration values measured in exposed values, sect 2.2) with respect non-transplanted samples (i.e. concentration values measured in unexposed samples, sect 2.2).

2.2 Terms and definition

For the purposes of this document the following terms and definitions apply.

- **B ratio (Bioaccumulation ratio)** ^N. The dimensionless ratio between species-specific element concentration values measured in native lichen samples and the corresponding background element concentration values (BECs).

-
- **Background area**^T. A remote, limited-extended, and environmentally homogeneous area, far from point and non-point sources of pollution, in which lichen thalli are collected for transplantation purposes.
 - **Background Element Concentration values (BECs)**. Species-specific element concentration values measured in lichen samples reflecting proximate-natural, unaltered conditions.
 - **Bioaccumulation**. Process whereby a substance present in the environment (in air, water or soil) accumulates at the surface of an organism and/or penetrates it. The accumulated compounds generally occur in the bioaccumulator organism at concentrations greater than those observed in the reference environmental matrix.
 - **Biomonitoring**. The set of techniques aimed at assessing the effects of pollution or other environmental changes on the biotic component of an ecosystem.
 - **Bulk lichen material**^T. The pool of suitable lichen thalli as derived by the collection in the background area.
 - **Epiphyte**. A plant or plant-like organism growing on another plant, dependent on mechanical support but not deriving nutrients from the plant upon which it grows.
 - **Exposed sample**^T. Sample derived by the random selection of thalli from the bulk lichen material, exposed for a known time span in the study area, and meant to be analysed for the assessment of the content of target elements.
 - **EU ratio (Exposed-to-Unexposed ratio)**^T. The dimensionless ratio between species-specific element concentration values measured in exposed lichen samples and the corresponding element concentration values measured in unexposed samples.
 - **Exposure device**^T. Every device supporting whole thalli or their portions during the transplant.
 - **Foliose lichen**. Leaf-like, flat lichen, adhering to the substrate.
 - **Fruticose lichen**. Shrubby, ascending or pendulous lichen attached to the substrate at its base.
 - **Lichen transplant**^T. Technique involving the collection of bulk lichen material in a background area, and the subsequent exposure of lichen samples for a certain time span in a target study area.
 - **Lichen**. Ecologically obligate, stable, self-supporting symbiotic association of a fungus (the mycobiont, generally an ascomycete) and one or more populations of green algae and/or cyanobacteria (the photobionts), resulting in a stable vegetative structure (“thallus”) with a definite morphology.
 - **Native lichen**^N. Lichen grown in a target study area.
 - **Non-point source of pollution**. The set of different extensive pollution sources (e.g. domestic heating, agricultural activities, vehicular traffic), in direct contrast to point sources of pollution.
 - **Non-sampleable areas**. Temporarily or permanently inaccessible areas (e.g. private areas for which access permission was temporarily/permanently denied, areas characterized by permanent physical barriers or dangerous conditions).
 - **Point source of pollution**. An identifiable source of air pollution characterized by negligible extent and approximable as a point in mathematical modeling.
 - **Probabilistic sampling**. Part of statistical practice concerned with the selection of individual observations, with known probability, intended to yield knowledge about a population of concern, especially for the purposes of statistical inference.
 - **Sampling Unit (SU)**. In this context, a Sampling Unit is a portion of the study domain (e.g. circular plot) in which lichen samples are collected (native) or exposed (transplants).
 - **Standard reference material (SRM)**. Certified material used to check the quality of analytical measurements (e.g. BCR 482 ‘*Pseudevernia furfuracea*’, IAEA-336 ‘*Evernia prunastri*’).
 - **Standard tree**^T. Tree colonized by lichen thalli of the selected species above 100 cm from the ground.
 - **Stratum (plural: strata)**. In the context of stratified sampling, a homogeneous statistical sub-population, here intended as determined by a stratification criterion (quantitative or qualitative variable) within a heterogeneous population.
 - **Study area**. The target area of the biomonitoring survey. It must be defined in detail in terms of geographical setting, extent, land use, anthropization and topography.
 - **Study domain**. A geographical area including all the sampleable parts of the study area. It may coincide with the latter in the case of completely sampleable study area.
 - **Substrate tree species**. Tree species hosting the lichen species selected for the biomonitoring study.
 - **Unexposed sample**^T. Sample derived by the random selection of thalli from the bulk lichen material and meant to be analysed for the assessment of the content of target elements in the background area.
-

2.3 Equipment

2.3.1 Field work preparation equipment

- *Maps*. The use of maps characterized by different scales is necessary: small-scale maps are needed for the representation of the survey area as a whole (e.g. 1:250,000, 1:100,000 on the basis of the extension of the study area); large-scale maps (1:25,000, 1:10,000, and 1:5,000) are essential to locate SUs. Useful thematic maps include vegetation and land use maps (e.g. Corine Land Cover, Bossard et al., 2000, <http://land.copernicus.eu/>), aerial imagery, and country planning maps.
- *Geographic Information System (GIS)*. System for storing, analysing and presenting spatial and geographic data.
- *Statistic software*. Algorithms for random sampling.

2.3.2 Field equipment

Lichen collection

- *Field sheets* (Annex D).
- *Maps*.
- *Compass-clinometer*.
- *GPS receiver*.
- *Stainless steel scalpel*.
- *Shears and lopping shears*.
- *Magnifying glass*.
- *Meter rule*.
- *Non-talc latex gloves*.
- *Paper bags*.
- *Ladder*.
- *Plastic bands*.
- *Camera*.
- *Inert containers* (e.g., Petri dishes).

2.3.3 Laboratory equipment

Selection of lichen material

- *Filter paper*.
- *Usual small laboratory equipment* (plastic and stainless tweezers, ceramic scissors and blades, etc.).
- *Stereomicroscope*.
- *Precision balance*.
- *Non-talc latex gloves*.

Storage

- *Silica gel*.
- *Laboratory vacuum sealer*.
- *Freezer (-20°C)*.
- *Sealable laboratory plastic or glass containers*.

Milling

- *Stove*.
- *Agate or ceramic mortar and pestles*.
- *Liquid nitrogen*.
- *Planetary ball mill*.
- *Stainless spatulas*.
- *Usual small laboratory equipment* (see supra).

Exposure device preparation^T

- *Wooden or stainless-steel rods*.

-
- *Lattice-work.*
 - *Fishing wire.*
 - *Plastic bands.*
 - *Usual small laboratory equipment (see supra).*

3. GUIDELINES FOR BIOACCUMULATION OF ELEMENTS USING NATIVE LICHENS

These guidelines describe the actions that shall be undertaken for the implementation of the method of the native lichens to monitor the bioaccumulation of macro- and trace elements. In particular, protocols and established procedures are described for all the phases of a biomonitoring survey based on native lichens, from the lichen sampling to the sample preparation for analytical determination of target elements (a workflow with the standard main phases of the method is reported in Annex A, Fig. A1). This chapter also includes specific indications for the expression of biomonitoring results, as well as an *ad hoc* tool for their proper interpretation.

3.1 Characterization of the study area

The study area has to be carefully characterized in terms of extent, land use, anthropization, topography, meteo-climatic conditions, point and non-point sources of pollution (sect. 2.2). All thematic maps and GIS (sect. 2.2) layers have to be as much as possible up-to-date and their reference source must always be specified. For the cartographic elaborations the selected reference system and coordinates shall always be specified, however the use of WGS 84 system and UTM projection is advisable.

The degree of heterogeneity of the study domain (sect. 2.2) can be evaluated on the basis of several variables such as the altitude, land use, density of resident population, vegetation etc; the ecological variables taken into account for such an assessment must be explicitly declared.

3.2 Sampling design

Given the use of a probabilistic sampling approach, different sampling designs can be adopted. The most appropriate design must be decided on the basis of the characteristics of the study area (degree of heterogeneity of the territory) and the study objective (sect. 2.1.2), taking into account the following aspects:

- The distribution of potentially suitable trees over the study area (i.e., the standard trees, sects. 2.2, 3.3.1), which should be as much known as possible, e.g. by using aerial imagery or analogous information sources, or by means of a preliminary inspection throughout the study area.
- The distribution of the selected lichen species over the study area.
- The diffusion models of the main pollutants affecting the study area, when available.
- The possible occurrence of areas with restricted access, such as private estates, military areas, etc., which should be preliminarily checked, in order to exclude possible non-sampleable areas (sect. 2.2) from the study domain and to define the target population accordingly.

Note. Examples of possible sampling methods and SU exclusion/inclusion criteria are reported in Annex F.

3.2.1 Sampling unit (SU)

The characteristics of the SUs (sect. 2.2) are selected on the basis of the distribution of potential suitable trees within the study areas. Two options are allowed:

Plot sampling: each plot represents a SU. Since the selected plot size depends on tree density in the study domain, different plot sizes could be considered in different biomonitoring surveys, but circular sampling plots with a radius ranging between 30 and 250 m are recommended. Within the same biomonitoring survey, plots should have the same shape and dimension. Each plot has to be centred in the coordinates of a previously selected sampling point.

Tree-based (plot-less) sampling: a SU is represented by a single tree or by a cluster of trees.

3.2.2 Sampling density

The minimum number of SUs has to be calculated on the basis of available sample size equations for different sampling designs (e.g., Elzinga et al., 2001). Usually, these formulae require preliminary information about the data variability in the study area, achievable by pilot studies and/or by reviewing data from previous campaigns (or by surveys carried out in comparable areas). In order to calculate the sampling density, further inputs are required to specify the required/desired precision.

3.2.3 Suitable lichen species

The use of a single lichen species within a study is recommended. All foliose and fruticose epiphytic lichens can potentially be considered. The choice shall be guided by various criteria, including:

- *Protection status*: the selected species must not be a protected species (IUCN Red List, or any other national or regional list of protection). The operator must be aware of the conservation status of the selected species before beginning the investigation.
- *Abundance*: the selected species must be sufficiently abundant in the study area to preserve the population and provide enough biomass for the chemical analyses (possibly, also for repeated surveys; case ii, sect. 2.1.2).
- *Ubiquity*: for studies conducted over very large areas the widespread presence of the selected species shall be as great as possible.
- *Identification easiness*: the selected species must be easy to identify in the field with minimal logistic means. Sampling requires personal having the necessary expertise. However, once in the laboratory, a further check based on suitable identification keys is suggested (see references in Annex B).
- *Availability of background element concentration values (BECs)*: it is suggested to select species with BEC values (sect 2.2) already available for the elements of interest. It is advisable to refer to Cecconi et al. (2018, 2019a, 2019b).

A brief description of the lichen species most commonly used in biomonitoring studies is reported in Annex B.

Note. In case it is necessary to use more than a single species for the same biomonitoring study, an inter-species comparison of element concentration bioaccumulated is recommended. In this case, samples of all the selected species should be collected at each SU. This applies only when BECs are not available for the selected species (or for the targeted elements).

3.3 Fieldwork

In the following section, the operations to be performed at each SU in the field are described, including the selection of standard trees, the collection and packaging of the lichen material for the transport to the laboratory.

3.3.1 Sample collection

Samples of the selected species can be collected on the trunk or on the branches of standard trees (sect. 2.2) above 100 cm from the ground to avoid terrigenous contamination. The collection of thalli growing on tree knots and on damaged, parasitized or decorticated parts of trunks should be avoided. It is also important not to simultaneously collect thalli from branches and trunks, since different positions on trees may affect their baseline composition (Adamo et al., 2008).

During field work, the following actions should be undertaken in each SU.

- **Perform a preliminary survey of the SU.** A preliminary survey must be performed to assess whether enough lichen material of the selected species is available and to check the number of standard trees in the SU.
- **Select the standard trees.** Standard trees must be selected for the lichen material collection. Fig. 3.1 schematically shows the main cases, in descending order (a-f) from the most to the less recommended option:
 - a) More than three standard trees (five in the example) are available in the SU and the amount of lichen material of the selected species is sufficient to obtain three analytical samples (sect. 3.4). The SU can be sampled. The material is collected from all the available standard trees.
 - b) More than three standard trees (five in the example) are available in the SU, but on the first three trees the amount of lichen material of the selected species is sufficient to obtain three analytical samples. The SU can be sampled. The material is collected from the three standard trees closest to the SU center.
 - c) Three standard trees are available in the SU, and the amount of lichen material of the selected species is sufficient to obtain three analytical samples. The SU can be sampled. The material is collected from the three available trees.
 - d) Two standard trees are available in the SU and the amount of lichen material of the selected species is sufficient to obtain three analytical samples. The SU can be sampled. The material is collected from the two available trees.

- e) A single standard tree is available in the SU and the amount of lichen material of the selected species is sufficient to obtain three analytical samples. The SU can be sampled. The material is collected from the available tree.
- f) One standard tree (at least) is available in the SU, but the amount of lichen material of the selected species is not sufficient to obtain three analytical samples. If the material is sufficient to obtain one or two samples, the SU can be sampled, but in that case the results of this SU should be considered with caution. If the material is not sufficient even to obtain a single sample, the SU cannot be sampled.
- g) There are no standard trees in the SU. The SU cannot be considered.

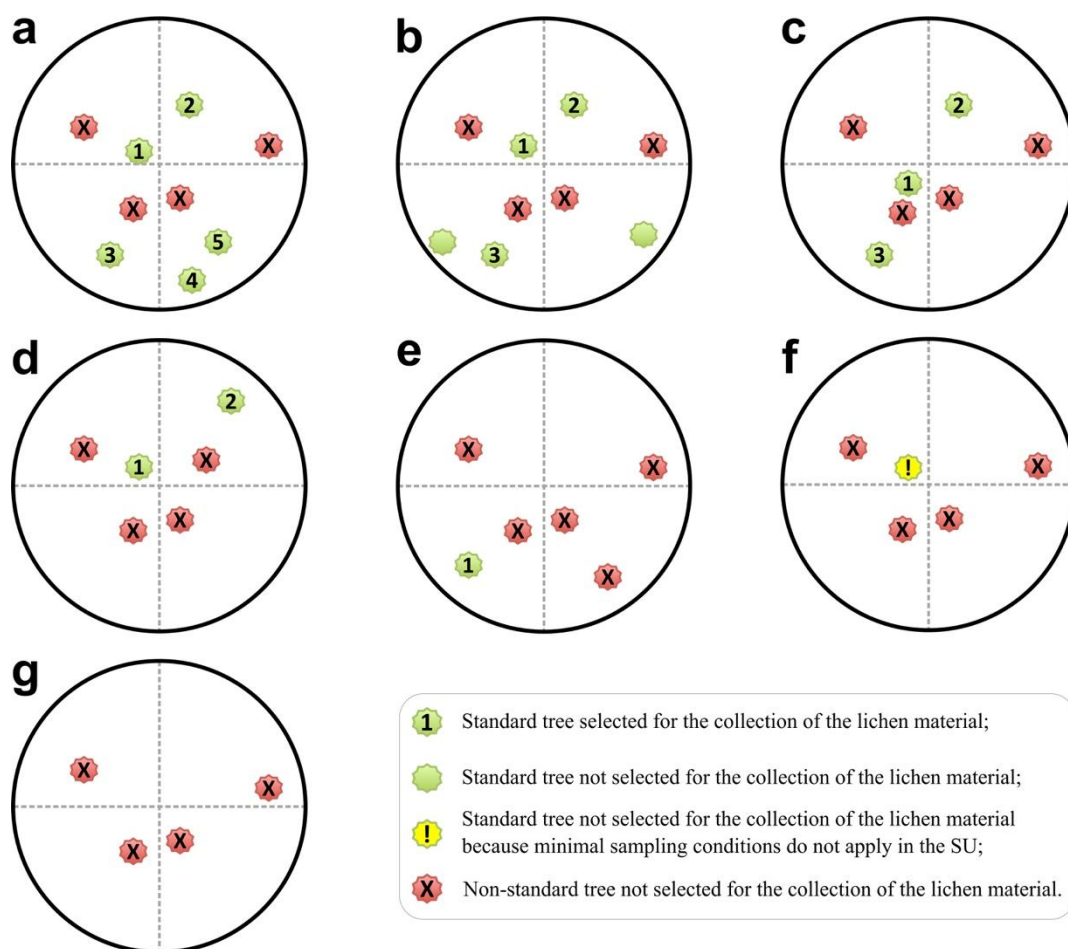


Figure 3.1 – Selection of standard trees for the collection of lichen material: casuistry.

- **Collect at least three samples of lichen material.** In this context, one sample is defined as the quantity of marginal parts of lichen thalli, sufficient to obtain an analytical sample of 200 mg. The samples must be collected at a height from the ground ≥ 100 cm. A maximum height limit is not set, as long as a safe collection procedure is guaranteed. Lichen samples must be collected using contaminant-free tools (e.g. a stainless steel or ceramic blade knife or scalpel), wearing non-talc gloves and being careful not to remove too much bark. Very small thalli (<1 cm of diameter), parts of thalli with fruiting bodies (apothecia) or clearly damaged thalli (for example thalli showing chlorosis or covered by algae) must be excluded from the collection.

Note. During the transport to the laboratory, the lichen samples must be stored in brand new, sealable, pre-labeled containers (e.g. paper envelopes, Petri dishes etc.).

3.4 Preparation of analytical samples

The following sections describe the operations to be performed in the laboratory, including sample cleaning, grinding and homogenization as well as the sample storage.

If the samples collected are damp, dry them in a protected environment, at room temperature (< 40°C) within 24h since the collection.

Before cleaning, the material must be kept dry, in a closed and clean environment. If cleaning cannot be performed within 7 days, the thalli must be stored in the freezer and then thawed at the time of cleaning, which will still be carried out within a year of freezing the sample.

3.4.1 Selection of suitable lichen material

The outermost portions of lichen thalli (without fruiting bodies, lichenicolous fungi or epiphytic algae) must be selected and cleaned from pieces of bark or other extraneous materials in order to properly obtain a sample. The length of outermost parts is species-specific, as depending on the size of thalli, shape and specific biomass of target taxon (Annex B). Outermost parts must be selected under a stereomicroscope by means of plastic or stainless tweezers.

This operation is particularly critical for the subsequent determination of the concentration of the elements in the sample and must therefore be carried out with particular attention. The samples have not to be washed to avoid losing particles trapped on the lichen surface and because there is evidence that the washing procedure can unpredictably alter the elemental composition of lichens (Bettinelli et al., 1996). Certain metals may accumulate in foliose lichens (sect 2.2) in zones according to age, i.e. exposure time (Hale & Loawrey, 1985; Bargagli et al., 1987; Nimis et al., 2001). For this reason, depending on the type of the study, different parts of the lichen samples could be considered: for example, in foliose lichens the outermost 3 mm of the thallus is physiologically the most active part and has an age of about one year (Tretiach & Carpanelli, 1992).

3.4.2 Sample grinding and homogenization

Each sample must be pulverized or finely chopped (i.e., homogenized). Pulverization can be carried out manually or with automated milling devices (e.g., ball mills; sect. 2.3.3).

Manual pulverization is carried out by soaking samples in c. 50 ml of liquid nitrogen and grinding them in ceramic or agate mortar.

The use of contaminant-free grinding materials is of primary importance. Particular attention should be also posed in cleaning jars, mortars and accessory instrumentation between subsequent milling cycles.

The pulverized material must be stored in contaminant-free, sealable, plastic or glass laboratory containers.

Note. After pulverization, the mass of a lichen sample should not be lower than 200 mg.

3.4.3 Dry weight determination

The twofold purpose of drying the pulverized samples is to protect them against microbial decomposition in case of subsequent storage, and to acquire a constant reference value, as opposed to the fresh weight. Pulverized or chopped samples have to be oven-dried at 105°C for at least 7 h to achieve a moisture content of about 8% (Quevauviller et al., 1996). Limited to volatile elements such as mercury, the determination of the dry weight should be performed at lower temperatures in order to prevent the possible loss of volatile mercury species. In this case, drying can be carried out at 40-50°C for 24 h (Markert, 1995). In addition, in case of surveys exclusively targeting mercury, the operators(s) shall consider the use of dedicated analyser systems such as Flow Injection Mercury Systems (Adamo et al., 2008) or combustion/trap technique (e.g., solid samples can be analysed by combustion and subsequent trapping of mercury on gold and analysis by Atomic Absorption Spectroscopy, with no need of wet acid mineralization; Roos-Barracough et al., 2002).

3.4.4 Sample storage

Analytical samples may be stored, making sure that the storage method and duration do not influence the elemental concentrations. Pulverized samples are generally sealed in plastic tubes or eppendorfs and stored away from heat sources until analytical determination.

Note. After storage at low temperature, the samples must be thawed in silica gel to avoid the formation of humidity. In case of volatile elements, the analytical determination must be carried out as soon as possible.

3.5 Analytical procedures

3.5.1 Chemical analysis

The analytical determination of target contaminants can be performed using several analytical techniques. Appointed laboratories must be accredited for the analyses of plant matrices, have proven expertise with complex matrices, and apply pertinent quality assurance measures (e.g. the analysis of SRMs). It is suggested to use a Standard Reference Material (SRM, sect. 2.2) specific for lichen matrices (e.g. BCR 482 ‘*Pseudevernia furfuracea*’, IAEA-336 ‘*Evernia prunastri*’; Stone et al., 1995; Quevauviller et al., 1996).

3.5.2 Digestion method

When required by the selected analytical technique, pulverized samples have to be mineralized by means of an acid digestion before the analytical determination. In particular, a ‘total’ digestion with hydrofluoric acid (e.g., HF/HNO₃/HCl: EN 13656; SW-846 EPA Method 3052) should be performed, since the addition of this strong acid generally ensures better recovery (and higher analytical accuracy) for elements present in aluminosilicates and other minerals typically resistant to HF-free acid attacks (Yafa & Farmer, 2006; Cecconi et al., 2019b).

3.6 Expression of results

The results of a biomonitoring survey are the element concentration values measured in lichen samples expressed as $\mu\text{g g}^{-1}$ dry weight (DW). The elemental content has to be reported as raw data (i.e., $n \geq 3$ concentration values per SU; sect. 3.3.2; Fig. 3.3). Besides the raw data, mean values and associated uncertainty for each SU have to be reported as well (Fig. 3.3).

In order to assess the elemental enrichment in a single SU, and for data interpretation purposes (sect. 3.7), the mean elemental concentrations measured in lichen samples have to be expressed with respect to background element concentration values (BECs, see definition in sect 2.2) in terms of the so-called *B* ratio (formula 3.1; definition in sect. 2.2; Cecconi et al., 2019a).

Methodologically uniform, species-specific national BECs for *Flavoparmelia caperata* and *Xanthoria parietina*, the most used species for biomonitoring applications with native lichens, are reported in Table 3.1 for two sets of 11 and 10 elements of environmental concern, respectively. For details on BEC construction, see Cecconi et al. (2019a).

Note. Methodologically uniform regional BECs are also available for the fruticose species *Pseudevernia furfuracea* for 43 elements. In this case, two sets of BECs were provided, respectively obtained by a total, HF-based digestion method (Cecconi et al., 2019b) and a partial, HF-free method (Cecconi et al., 2018).

Table 3.1 – Background element concentration values for species of common use in biomonitoring by native lichens. BECs for *Flavoparmelia caperata* and *Xanthoria parietina* and associated uncertainty (Δ_{BEC}) expressed in $\mu\text{g g}^{-1}$. The uncertainty associated to BEC data is expressed in terms of standard deviation (n.a., data not available).

Element	<i>Flavoparmelia caperata</i>		<i>Xanthoria parietina</i>	
	BEC	Δ_{BEC}	BEC	Δ_{BEC}
Al	253	37	372	59
As	0.18	0.03	0.15	0.02
Cd	0.18	0.03	0.07	0.01
Cr	1.17	0.24	1.61	0.28
Cu	6.2	0.9	4.5	0.3
Hg	0.057	0.021	0.035	0.009
Ni	1.27	0.20	1.64	0.22
Pb	2.37	0.45	1.00	0.21
Ti	19.5	5.8	37.3	7.3
V	0.75	0.11	n.a.	n.a.
Zn	35.3	4.3	21.3	2.6

For the i^{th} SU ($i = 1, \dots, N$) and a generic element y , for which n measurements are available ($j = 1, \dots, n$), the mean value of the B ratio is given by:

$$B^{(i)} = \frac{y^{(i)}}{y_{BEC}} \quad (3.1)$$

$$y^{(i)} = \frac{1}{n} \sum_{j=1}^n y_j^{(i)} : \text{mean of the } n \text{ concentration values measured in samples of the } i^{\text{th}} \text{ SU } (n \geq 3);$$

y_{BEC} : species-specific national BEC value (Table 3.1).

3.7 Interpretation of results

In order to evaluate the magnitude of pollution phenomena in a study area, the biomonitoring results must be subjected to adequate statistical analysis. Besides, the results expressed as B ratios (sects. 2.2 and 3.6) have to be carefully interpreted by means of *ad hoc* interpretative tools.

3.7.1 Basic statistical analyses of biomonitoring data

The results of a biomonitoring survey (either expressed as mean element concentration or B ratio for each SU, formula 3.1) can be analysed by means of univariate and multivariate statistics, and non-parametric models (Brunialti & Frati, 2007). Data, in the form of matrices of elemental concentrations in the SUs, are generally analysed with statistical techniques of consolidated use for the identification of emission sources, such as correlation analysis, cluster and principal component analyses (Bargagli & Mikhailova, 2002). Moreover, bioaccumulation data can be represented through two-dimensional maps by means of suitable mapping software (Annex H).

Statistical tools and mapping procedures should allow to identify (i) spatial patterns of element content in lichen samples, (ii) temporal trends of elemental content in case of repeated surveys, and (iii) terrigenous contamination of samples due to soil resuspension phenomena.

Note. Terrigenous contamination can be punctually evaluated by means of the so-called Enrichment Factor (EF), which compares the relative concentration of an analyte accumulated in lichen samples to that in soil samples (Bargagli & Mikhailova, 2002). In this case, the soil sampling must follow specific guidelines (e.g., Icp-forests.net) and soil samples have to be analysed with the same analytical technique used for lichens.

3.7.2 Interpretative tool: the bioaccumulation scale for native lichens

An interpretative scale is a fundamental tool to properly assess the severity of pollution phenomena. A ‘Bioaccumulation Scale’ for biomonitoring results by native lichens is provided in Cecconi et al. (2019a) and

here reported in Table 3.2. The scale relies on the analysis of the distribution of hundreds of B ratio records obtained from the literature and consists in five classes corresponding to increasing levels of bioaccumulation in native lichens. Lower and upper limits of classes refer to values corresponding to 25th, 75th, 90th and 95th percentiles of the B ratio distribution (for details, see Cecconi et al., 2019a).

Table 3.2 – *Bioaccumulation scale for native lichens.*

Bioaccumulation scale with classes, descriptions, percentile thresholds and the corresponding B ratio values, as well as RGB and HTML colour codes to be associated to bioaccumulation classes.

Bioaccumulation class		Percentile thresholds	B ratio	Colour code	
ID	Description			RGB	HTML
1	Absence of bioaccumulation	$\leq 25^{\text{th}}$	≤ 1.0	0, 0, 255	#0000FF
2	Low bioaccumulation	$(25^{\text{th}}, 75^{\text{th}}]$	$(1.0, 2.1]$	0, 128, 0	#008000
3	Moderate bioaccumulation	$(75^{\text{th}}, 90^{\text{th}}]$	$(2.1, 3.4]$	255, 243, 15	#FFF30F
4	High bioaccumulation	$(90^{\text{th}}, 95^{\text{th}}]$	$(3.4, 4.9]$	255, 0, 0	#FF0000
5	Severe bioaccumulation	$> 95^{\text{th}}$	> 4.9	128, 0, 64	#800040

3.7.3 Attribution of the bioaccumulation class to the Sampling Unit

The attribution of the bioaccumulation class (Table 3.2) to a SU has to be performed on the basis of the mean value of the B ratio (formula 3.1, sect. 3.6) diminished by its uncertainty $\Delta(B^{(i)})$. Thus, for the i^{th} SU, the corresponding bioaccumulation class is determined on the basis of $B^{(i)} - \Delta(B^{(i)})$ (Fig. 3.2), where $\Delta(B^{(i)})$ is obtained by propagating the uncertainty associated to $y^{(i)}$ and y_{BEC} (see sect. 3.6 for definitions).

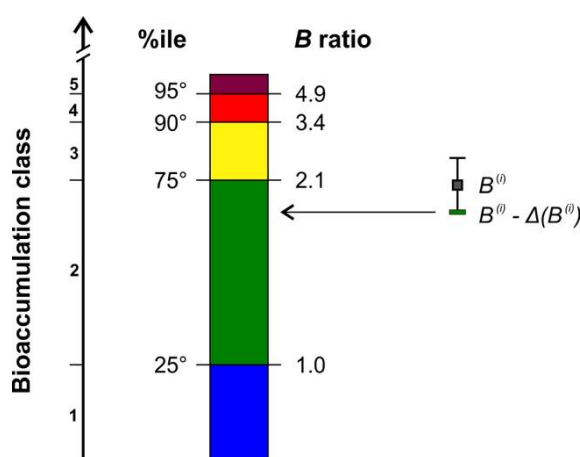


Figure 3.2 – *Way of attribution of the bioaccumulation class to a SU.*

Percentile thresholds and corresponding B ratio values of the bioaccumulation scale for native lichens.

In order to calculate $\Delta(B^{(i)})$, the operator must know: (i) the uncertainty associated to the element-specific BEC value, i.e. $\Delta(y_{BEC})$, and (ii) the uncertainty associated to the mean element content value revealed in the i^{th} SU, i.e. $\Delta(y^{(i)})$. The former is provided in Table 3.1, and the latter can be calculated by assuming a triangular distribution for element concentration data, since this distribution is widely used in ecology to calculate the uncertainty of measurement in case of limited sample size (Physics.nist.gov). In particular, $\Delta(y^{(i)})$ is obtained by calculating the difference between the maximum and the minimum concentration values within the SU and dividing such a difference by $\sqrt{6}$ (formula 3.2, with coverage factor $k = 2$, that guarantees 95% confidence limits).

$$\Delta(y^{(i)}) = k \left(\frac{\max\{y_j^{(i)}\} - \min\{y_j^{(i)}\}}{2\sqrt{6}} \right) = \frac{\max\{y_j^{(i)}\} - \min\{y_j^{(i)}\}}{\sqrt{6}} \quad (3.2)$$

$\max\{y_j^{(i)}\}$: maximum among the n concentration values measured in samples of the i^{th} SU;

$\min\{y_j^{(i)}\}$: minimum among the n concentration values measured in samples of the i^{th} SU.

Finally, $\Delta(B^{(i)})$ can be easily calculated according to formula 3.3.

$$\Delta(B^{(i)}) = B^{(i)} \sqrt{\left(\frac{\Delta(y^{(i)})}{y^{(i)}}\right)^2 + \left(\frac{\Delta(y_{BEC})}{y_{BEC}}\right)^2} \quad (3.3)$$

Numerical example

Suppose one wants to assess the bioaccumulation level of nickel (Ni) in *Flavoparmelia caperata* samples in $N = 5$ SUs in a study area, having $n = 3$ samples in each SU (Fig. 3.3). For generalization purposes, mean Ni concentrations in the SUs and the corresponding BEC value are referred as y and y_{BEC} , respectively (Fig. 3.3).

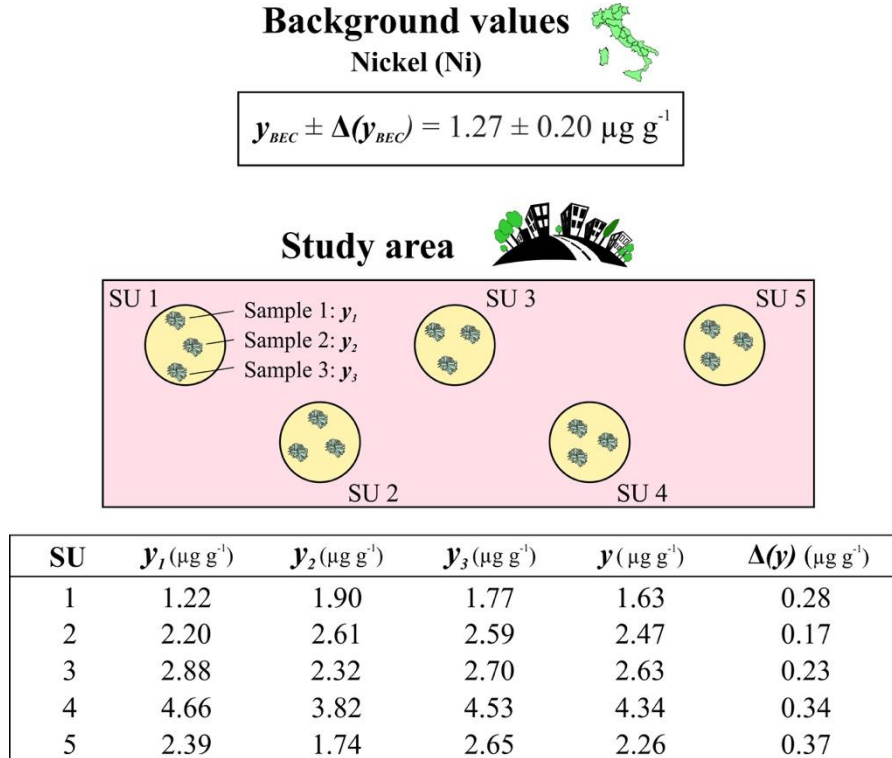


Figure 3.3 – Numerical example: initial data.

Mean BEC value and its associated uncertainty (y_{BEC} and $\Delta(y_{BEC})$) for the element Ni (Table 3.1), as well as Ni concentration values (y_j) measured in $n = 3$ samples collected at each of the $N = 5$ SUs, their mean values and associated uncertainties (y and $\Delta(y)$).

From the exemplifying dataset in Fig. 3.3, the application of formulae 3.1 - 3.3 easily allows to calculate the mean B ratio, the associated uncertainty, and their difference ($B - \Delta(B)$), thus permitting the attribution of bioaccumulation classes to SUs (Table 3.2; Fig. 3.4).

SU	B	$\Delta(B)$	$B - \Delta(B)$	Class
1	1.28	0.30	0.99	1
2	1.94	0.33	1.61	2
3	2.07	0.37	1.70	2
4	3.41	0.60	2.81	3
5	1.78	0.41	1.37	2

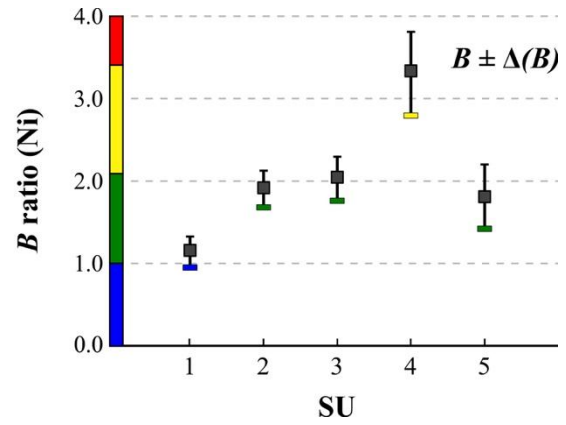


Figure 3.4 – Numerical example: final output.

B ratios and their associated uncertainties for the element Ni in 5 SUs. Their difference, used to assess the class for each SU is also reported, along with a graphic representation of the output.

4. GUIDELINES FOR BIOACCUMULATION OF ELEMENTS USING LICHEN TRANSPLANTS

These guidelines describe the actions that shall be undertaken for the implementation of the method of the lichen transplants to monitor the bioaccumulation of macro- and trace elements. In particular, protocols and established procedures are described for all the phases of a transplant-based biomonitoring survey, from the lichen collection in a background area, their transplant in a study area, to the sample preparation for analytical determination of target elements (a workflow with the standard main phases of the method is reported in Annex A, Fig. A2). This chapter also includes specific indications for the expression of biomonitoring results, as well as an *ad hoc* tool for their proper interpretation.

4.1 Lichen exposure in the study area: planning

4.1.1 Characterization of the study area

The study area has to be carefully characterized in terms of extent, land use, anthropization, topography, meteo-climatic conditions, point and non-point emission sources. All thematic maps and GIS (sect. 2.2) layers have to be as much as possible up-to-date and their reference source must always be specified. For the cartographic elaborations the selected reference system and coordinates shall always be specified, however the use of WGS 84 system and UTM projection is advisable. A proper characterization of the study area should allow the mapping of non-sampleable areas (sect. 2.2).

4.1.2 Sampling design

In the context of lichen transplants, samples are exposed in the study area for a defined time period (i.e., exposure time span; sect. 4.1.5) according to a given probabilistic-based exposure design (henceforth, sampling method; Annex F).

The exposure sites (i.e., the Sampling Units, SUs, sect 2.2) are identified by a pair of geographic coordinates corresponding to the centre of a circular plot. SUs, and samples exposed therein, must be labelled with unambiguous alphanumeric identification codes.

The choice of the sampling method has to be driven by the characteristics of the study area, the type of emission source and/or by estimates of the maximum accuracy achievable under the condition of fixed costs (Elzinga et al., 2001).

Note. Examples of possible sampling methods and SU exclusion/inclusion criteria are reported in Annex F.

4.1.3 Recommendations for sample exposure in the SUs

A minimum of three lichen samples have to be exposed within each SU. A sample corresponds to a certain number of thalli suitable to obtain enough mass for analytical determination (cfr. sects. 4.3.2 and 4.5.2). Thalli needed for one sample can be either transplanted as they are or secured to an exposure device (sects. 2.2, 2.3.3, 4.3.2, Annex C). When using exposure devices, a single device must support a number of thalli suitable to make up a single sample, thus for the exposure of three samples in a SU, three exposure devices are needed.

In a SU, samples (or exposure devices bearing samples) have to be installed on field supports, which can be either trees or artificial supports (e.g. lampposts or other purposely-made supports). In any case, samples have to be placed between 2 and 4 m above the ground, far from potential micro-contamination sources (e.g. gutter drains, roofs and electric cables), and the exposure methodology has to be kept constant within the same survey, that is, same kind of field support (either trees or artificial ones), same kind of exposure device and constant height above the ground. The cardinal orientation (N, S, W, E) of samples or exposure devices on their field supports should be randomly assessed. In case of scarcity of natural or artificial supports in a SU, lichen samples must be anyway placed at a minimum distance of 3 meters among each other (Annex C, Fig. C1-e). When tree branches are used as supports, samples can be exposed either out of the tree crown or under the crown, always keeping care in guaranteeing exposure uniformity within the study area.

4.1.4 Estimate of the sampling effort in the background area

In order to assess the sampling effort in the background area (see definition in sect. 2.2), the operator(s) must consider the study objective (sect. 2.1.2), the extension of the study area and the overall number of SUs (sect. 4.1.1), as well as the number of samples to be exposed in a single SU and the number of unexposed samples to be analysed (see definitions of ‘exposed and ‘unexposed’ sample in sect. 2.2). In this respect, as a rule of thumb, at least $n = 3$ samples have to be exposed in a single SU (sect. 4.1.3, 4.3.2), whereas $m > n$ samples have to be analysed for the assessment of the elemental content before the exposure (sects. 4.2.2, 4.3.2, 4.7, Annex G).

In general, the amount of collected lichen material should be enough to (i) assess the variability of elemental composition of the source lichen population (when needed; sect. 4.2.2 and Annex G), (ii) properly estimate an averaged element concentration in unexposed samples, and (iii) assemble at least three lichen samples for the exposure in a SU (sect. 4.3.2, Annex C).

4.1.5 Exposure duration

The suggested time span for a single exposure campaign ranges between 4 and 12 weeks (cfr. sect. 4.8.2). The week must be always used as the base time unit for the quantification of the exposure time span. In the case of repeated surveys, the exposure duration must be kept constant.

4.1.6 Ancillary information

It is strongly recommended to gather data on meteorological conditions and air pollutant concentration levels before and during the biomonitoring survey, since these can be helpful for the interpretation of bioaccumulation patterns.

4.2 Collection of the lichen material

4.2.1 Suitable lichen species

In the framework of this standard, both foliose and fruticose epiphytic lichen species (see definitions in sect. 2.2) can be used. However, the use of epiphytic fruticose taxa is recommended because such a growth form ensures higher biomass per lichen thallus than foliose species. Moreover, fruticose lichens are easier to clean, select, and install on exposure devices, therefore their use generally reduces processing time, enhances sample homogeneity and data accuracy (Wolterbeek & Bode, 1995). A list of suitable lichen species subdivided on the basis of their growth form is provided (see Annex B for a detailed description).

Since different lichen species may differ for both background element concentrations and accumulation capacity, the use of a single species within a study is mandatory. The species choice shall be guided by the following criteria:

- *Protection status*: the selected species must not be a protected species (IUCN Red List, or any other national or regional list of protection). The operator must be aware of the conservation status of the selected species before beginning the investigation.
- *Abundance*: the selected species must be sufficiently abundant in the background area (sect 2.2) to preserve the population and possibly to provide enough biomass for the for repeated surveys (case ii, sect. 2.1.2).
- *Identification easiness*: the selected species must be easy to identify in the field with minimal logistic means. Sampling requires personal having the necessary expertise. However, once in the laboratory, a further check based on suitable identification keys is suggested (see references in Annex B).

4.2.2 Characterization of the background area

The background area (sect 2.2) should be as much as possible ecologically and environmentally homogeneous. The environmental characterization of such area has to be performed by a preliminary field inspection (an example of field sheet is provided in Annex D, Fig. D2) and thematic maps of land use, vegetation, canopy cover, climate, altitude (digital elevation models), and lithology. In addition, the occurrence of poleosensitive lichen taxa can also be assessed to provide further evidence that the area is not affected by major contamination phenomena.

Preliminary assessment of elemental content variability in the background area

When a background area is selected for the first time, a pilot study is needed to assess the variability of the elemental content in the source lichen population. To this purpose, in Annex G a resampling-based method is proposed to provide criteria to assess whether an area can or cannot be considered as “a background” one. Contextually, the method is suitable to establish the minimum number of unexposed samples to obtain a reliable reference value (mean elemental content in the background area).

Note. Once the element content variability of the source lichen population has been assessed, the number of unexposed samples to be analysed can be kept constant for further biomonitoring surveys.

A good practice also consists in comparing the mean element content of unexposed samples with species-specific background estimates (if available) or, alternatively, to previously published values referring to the same species collected in other background areas, as selected on the basis of geographical proximity criterion.

These procedures are necessary to prevent the use of lichen thalli that, although collected in remote areas, can be characterized by element concentrations far above a national background reference, due to geochemical anomalies and/or long-range pollution transport (Fрати et al., 2005).

4.2.3 Procedures for lichen collection

Lichen thalli have to be collected on bark of either trunks or branches of healthy substrate trees, standing or with subvertical inclination. In order to enhance sample homogeneity and to avoid terrigenous contamination, thalli must be collected above 100 cm from the ground, and possibly by selecting a single substrate tree species (sect. 2.2). The collection of thalli growing on tree knots, on damaged or decorticated parts of trunks, as well as on parts parasitized by fungi or insects should be avoided. It is also important not to simultaneously collect thalli from branches and trunks, since different positions on trees may affect their baseline composition (Adamo et al., 2008).

Thalli have to be collected from trees using different tools and precautions on the basis of the growth form of target species.

- **From tree trunk.** Detach bark fragments bearing thalli by means of stainless knife or scalpel, taking care in minimizing the damage for substrate tree species.
- **From branches.** Cut portions of tree branches bearing lichen thalli by means of stainless tools.

In the field, any useful information about lichen collection sites in the background area (sect 2.2) should be recorded by the operators on field sheets (Annex D, Fig. D1).

Note. During the transport to the laboratory, the lichen material must be stored in brand new, sealable, pre-labeled containers (e.g. paper envelopes, Petri dishes etc.).

4.2.4 Morphometric characterisation of the lichen material

The elemental content in samples primarily depends on the entrapment of air particulate matter thanks to specific morphological features of thalli. Therefore, in order to enhance the morphological homogeneity of the bulk material, a visual morphometric characterization of thalli should be performed both in the field and in the laboratory. In particular, the selection of thalli for the exposure should be limited to those characterized by comparable size, branching degree and roughness of the external surfaces (e.g. presence/absence of reproductive structures).

4.3 Laboratory procedures

The following sections describe the operations to be performed in the laboratory before the exposure of samples in the study area.

4.3.1 Preparation of bulk lichen material

Bulk lichen material (see definition in sect. 2.2) consists of a pool of thalli collected in the background area (sect 2.2) and processed according to the following steps before their exposure in the study area.

- **Drying.** Thalli have to be air-dried in a protected environment at room temperature (< 40°C). Once adopted, procedure for drying should not be modified for subsequent surveys.

-
- **Cleaning.** Air-dried thalli have to be cleaned from extraneous materials (e.g. other lichen and moss species, soil debris, arthropods etc.) by using powder free gloves and proper tools (e.g. plastic or stainless tweezers etc., sect. 2.3.3).
Note. Thalli must not be washed or subjected to any other pre-treatment possibly affecting elemental content (Bettinelli et al., 1996).
 - **Storage.** Bulk lichen material can be either immediately processed to compose lichen samples (sect. 4.3.2) or stored for later use. Thalli can be kept dried at room temperature up to two weeks. Alternatively, thalli can also be stored for longer periods after treatment in silica-gel for 48h (relative water content < 5 -10%) and vacuum-packing; so-treated thalli can be stored up to 24 months at -20 ± 2 °C (Honegger, 2003). After prolonged storage, it is highly recommended to test the vitality of lichen thalli by routine chlorophyll fluorescence assay (e.g., maximum quantum yield of primary photochemistry of the lichen photobiont, F_v/F_m ; Jensen, 2002).

4.3.2 Sample preparation

A sample consists of a certain number of thalli randomly selected from the bulk lichen material (sect. 2.2). The suitable number of thalli needed to make up a single sample is species-specific and depends on size, shape and specific biomass of the target taxon. During sample preparation, the operator shall consider the loss of lichen material due to (a) sample processing (sect. 4.5.1 and 4.5.2) and (b) the field exposure in the SUs.

Samples can be divided in:

- **Unexposed samples.** Unexposed samples can be analysed either (i) before the beginning of lichen exposure in the study area or (ii) at the end of the biomonitoring survey, along with exposed samples. In case (i) unexposed samples have to be processed according to sect. 4.5 and analysed according to sect. 4.6; in case (ii), unexposed samples have to be stored according to sect. 4.3.1 until their processing that will be performed contextually to that of exposed samples.
- **Samples for field exposure.** As specified in sect. 4.1.3, lichen samples can be either exposed as they are (still attached to a piece of their natural substrate, i.e., twig or trunk bark) or by means of dedicated exposure devices. In any case, prior to their field exposure / assembly on exposure devices, twigs or bark fragments bearing thalli have to be reduced to appropriate size in order to ensure easier assembling on field supports / exposure devices.

Twigs and bark fragments may be secured on field supports / exposure devices by means of plant mastics, plastic bands or any other suitable material (Annex C, Fig. C1-a - C1-g).

Note. Once prepared, samples for field exposure, either mounted or not on exposure devices, have to be adequately packed in order to avoid any potential contamination before their exposure, that must be in any case performed within two weeks (in this period, samples must be kept dry at room temperature).

4.4 Installation and retrieving of samples

During the operations of exposure and retrieving of samples in the field (study area), any useful information related to exposure conditions (e.g. potential changes with respect to what noticed during preliminary inspections) should be recorded by the operators on field sheets (Fig. D3, Annex D).

- **Installation.** During the installation of samples / exposure devices, the operator should pay particular attention in replicating as much as possible the natural orientation of lichen surfaces, since these organisms are dorsiventrally organized.
- **Retrieving.** After the exposure, samples have to be retrieved and placed in brand new paper envelopes labelled with the identification code of SUs.

4.5 Laboratory preparation of analytical samples

Once in the laboratory, both unexposed and exposed samples will be singly processed by selecting and consecutively grinding only the outermost parts of thalli (Annex B), in order to enhance the homogeneity of the material. The selected portions will compose the samples meant to be subjected to multi-element analysis (sect. 4.6.1).

4.5.1 Selection of suitable lichen material

The outermost portions selected from thalli have to be free of fruiting bodies, not infected by lichenicolous fungi or covered by epiphytic algae. The length of outermost parts is species-specific, as depending on the size of thalli, shape and biomass of the selected species (Annex B). Outermost parts have to be selected under a stereomicroscope with the help of plastic or stainless tweezers.

This operation is particularly critical for the subsequent determination of elemental concentration in the sample and must therefore be carried out with particular attention.

Note. Even at this stage, samples must not be washed to avoid losing particles trapped on the lichen surface and alter the elemental composition.

4.5.2 Sample grinding and homogenization

Each sample has to be pulverized or finely chopped (i.e., homogenized). Pulverization can be carried out manually or with automated milling devices (e.g., ball mills; sect. 2.3.3).

Manual pulverization is carried out by soaking samples in c. 50 ml of liquid nitrogen and grinding them in ceramic or agate mortar.

The use of contaminant-free grinding materials is of primary importance. Particular attention should be also posed in cleaning jars, mortars and accessory instrumentation between subsequent milling cycles.

The pulverized material has to be stored in contaminant-free, sealable, plastic or glass laboratory containers.

Note. After pulverization, the mass of a lichen sample should not be lower than 200 mg.

4.5.3 Dry weight determination

The twofold purpose of drying the pulverized samples is to protect them against microbial decomposition in case of subsequent storage, and to acquire a constant reference value, as opposed to the fresh weight. Pulverized or chopped samples have to be oven-dried at 105°C for at least 7 h to achieve a moisture content of about 8% (Quevauviller et al., 1996). Limited to volatile elements such as mercury, the determination of the dry weight should be performed at lower temperatures in order to prevent the possible loss of volatile mercury species. In this case, drying can be carried out at 40-50°C for 24 h (Markert, 1995). In addition, in case of surveys exclusively targeting mercury, the operators(s) shall consider the use of dedicated analyser systems such as Flow Injection Mercury Systems (Adamo et al., 2008) or combustion/trap technique (e.g., solid samples can be analysed by combustion and subsequent trapping of mercury on gold and analysis by Atomic Absorption Spectroscopy, with no need of wet acid mineralization; Roos-Barraclough et al., 2002).

4.5.4 Sample storage

Samples may be stored, making sure that the storage method and duration do not influence the elemental concentrations. Analytical samples are generally sealed in plastic tubes or eppendorfs and stored away from heat sources until analytical determination.

Note. After storage at low temperature, the samples must be thawed in silica gel to avoid the formation of humidity. In case of volatile elements, the chemical analyses must be carried out as soon as possible.

4.6 Analytical procedures

4.6.1 Chemical analyses

The analytical determination of target contaminants can be performed using several analytical techniques. Appointed laboratories must be accredited for the analyses of plant matrices, have proven expertise with complex matrices, and apply pertinent quality assurance measures (e.g. the analysis of SRMs). It is suggested to use a Standard Reference Material (SRM, sect. 2.2) specific for lichen matrices (e.g. BCR 482 ‘*Pseudevernia furfuracea*’, IAEA-336 ‘*Evernia prunastri*’; Stone et al., 1995; Quevauviller et al., 1996).

4.6.2 Digestion method

When required by the selected analytical technique, pulverized samples have to be mineralized by means of an acid digestion before the analytical determination. In particular, a ‘total’ digestion with hydrofluoric acid (e.g., HF/HNO₃/HCl: EN 13656; SW-846 EPA Method 3052) should be performed, since the addition of this strong acid generally ensures better recovery (and higher analytical accuracy) for elements present in aluminosilicates and other minerals typically resistant to HF-free acid attacks (Yafa & Farmer, 2006; Cecconi et al., 2019b).

4.7 Expression of results

The results of a biomonitoring survey are the element concentration values measured in both unexposed and exposed lichen samples, and expressed as $\mu\text{g g}^{-1}$ dry weight (DW). The elemental content has to be reported as raw data, i.e., $n \geq 3$ concentration values for exposed samples per SU, and $m > n$ concentration values of unexposed samples (sect. 4.1.4; Fig. 4.2). Besides the raw data, mean values and associated uncertainties have to be reported as well (Fig. 4.2).

In order to assess the elemental enrichment in a single SU, and for data interpretation purposes (sect. 4.8), the mean elemental concentrations measured in ‘Exposed’ samples (E) have to be expressed with respect to those observed in ‘Unexposed’ samples (U), in terms of the so-called EU ratio (formula 4.1; definition in sect. 2.2; Cecconi et al., 2019a).

For the i^{th} SU ($i = 1, \dots, N$) and a generic element y , for which n measurements are available for exposed samples ($j = 1, \dots, n$), and $m > n$ measurements are available for unexposed samples ($j = 1, \dots, m$), the mean value of the EU ratio is given by:

$$EU^{(i)} = \frac{y_E^{(i)}}{y_U} \quad (4.1)$$

$y_E^{(i)} = \frac{1}{n} \sum_{j=1}^n y_{Ej}^{(i)}$: mean of the n concentration values measured in samples exposed in the i^{th} SU ($n \geq 3$);

$y_U = \frac{1}{m} \sum_{j=1}^m y_{Uj}$: mean of the m concentration values measured in unexposed samples collected in a consolidated background area ($m > n$).

4.8 Interpretation of results

In order to evaluate the magnitude of pollution phenomena in a study area, the biomonitoring results must be subjected to adequate statistical analysis, and EU ratios (sects. 2.2 and 4.7) have to be carefully interpreted by means of *ad hoc* interpretative tools.

4.8.1 Basic statistical analyses of biomonitoring data

The results of a biomonitoring survey (either expressed as mean element concentration or EU ratio for each SU, formula 4.1) can be analysed by means of univariate and multivariate statistics and non-parametric models (Brunialti & Frati, 2007). Data, in the form of matrices of elemental concentrations in the SUs, are generally analysed with statistical techniques of consolidated use for the identification of emission sources, such as correlation analysis, cluster and principal component analyses (Bargagli & Mikhailova, 2002). Moreover, bioaccumulation data can be represented through two-dimensional maps by means of suitable mapping software (Annex H).

Statistical tools and mapping procedures should allow to identify (i) spatial patterns of element content in lichen samples, (ii) temporal trends of elemental content in case of repeated surveys, and (iii) terrigenous contamination of samples due to soil resuspension phenomena.

Note. Terrigenous contamination can be punctually evaluated by means of the so-called Enrichment Factor (EF), which compares the relative concentration of an analyte accumulated in lichens to that in soil (Bargagli & Mikhailova, 2002). In this case, the soil sampling must follow specific guidelines (e.g., Icp-forests.net) and samples have to be analysed with the same analytical technique used for lichens.

4.8.2 Interpretative tool: the bioaccumulation scale for lichen transplants

An interpretative scale is a fundamental tool to properly assess the severity of pollution phenomena. A ‘Bioaccumulation Scale’ for biomonitoring results by lichen transplants is provided in Cecconi et al. (2019a) and here reported in Table 4.1. The scale relies on the analysis of the distribution of hundreds of *EU* ratio records obtained from the literature, and refers to exposure time spans of 4, 8 and 12 weeks. The scale consists of five classes corresponding to increasing levels of bioaccumulation in transplanted lichen samples. Lower and upper limits of classes refer to values corresponding to 25th, 75th, 90th and 95th percentiles of the *EU* ratio distributions (for details, see Cecconi et al., 2019a).

Table 4.1 – Bioaccumulation scale for lichen transplants.

Bioaccumulation scale with classes, descriptions, percentile thresholds and the corresponding *EU* ratio values for three different exposure time spans (4, 8 and 12 weeks), as well as RGB and HTML colour codes to be associated to bioaccumulation classes.

Bioaccumulation class		Percentile thresholds	<i>EU</i> ratio			Colour code	
ID	Description		4 weeks	8 weeks	12 weeks	RGB	HTML
1	Absence of bioaccumulation	≤ 25 th	≤ 1.0	≤ 1.0	≤ 1.0	0, 0, 255	#0000FF
2	Low bioaccumulation	(25 th , 75 th]	(1.0, 1.8]	(1.0, 1.9]	(1.0, 1.8]	0, 128, 0	#008000
3	Moderate bioaccumulation	(75 th , 90 th]	(1.8, 2.5]	(1.8, 2.7]	(1.9, 3.1]	255, 243, 15	#FFF30F
4	High bioaccumulation	(90 th , 95 th]	(2.5, 2.8]	(2.7, 3.5]	(3.1, 3.7]	255, 0, 0	#FF0000
5	Severe bioaccumulation	> 95 th	> 2.8	> 3.5	> 3.7	128, 0, 64	#800040

4.8.3 Attribution of the bioaccumulation class to the Sampling Unit

The attribution of a SU to a class of the bioaccumulation scale (Table 4.1) has to be performed on the basis of the mean value of the *EU* ratio (formula 4.1, sect. 4.7) diminished by its uncertainty $\Delta(EU^{(i)})$. Thus, for the *i*th SU, the corresponding bioaccumulation class is determined on the basis of $EU^{(i)} - \Delta(EU^{(i)})$ (Fig. 4.1), where $\Delta(EU^{(i)})$ is obtained by propagating the uncertainty associated to $y_E^{(i)}$ and y_U (see sect. 4.7 for definitions).

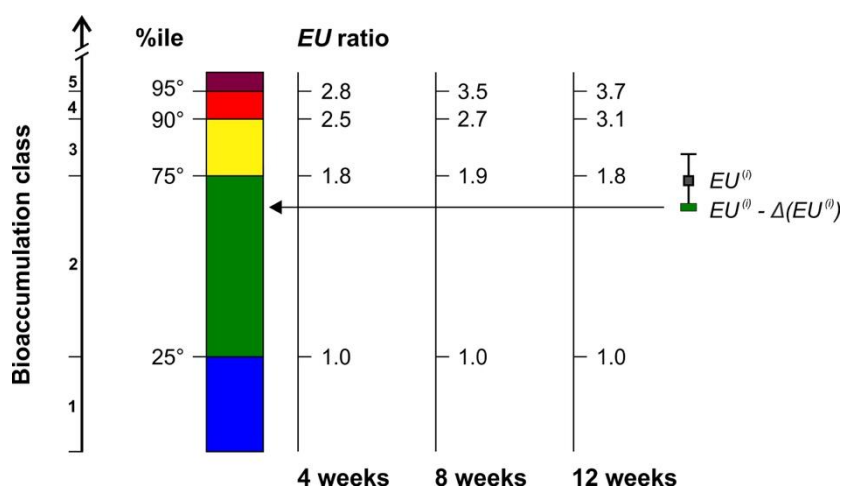


Figure 4.1 – Way of attribution of the bioaccumulation class to a SU.

Percentile thresholds and corresponding *EU* ratio values of the bioaccumulation scale for lichen transplants, with data separately reported for three different exposure time spans (4, 8 and 12 weeks).

In order to calculate $\Delta(EU^{(i)})$, the operator must know: (i) the uncertainty associated to the mean element concentration value in exposed samples in the i^{th} SU ($\Delta(y_E^{(i)})$), and (ii) the uncertainty associated to the mean unexposed element concentration value ($\Delta(y_U)$). Such uncertainties can be calculated by assuming a triangular distribution for element concentration data, since this distribution is widely used in ecology to calculate the uncertainty of measurement in case of limited sample size (Physics.nist.gov). In particular, these uncertainties are obtained by calculating the difference between the maximum and the minimum concentration values and dividing such a difference by $\sqrt{6}$ (formulae 4.2 and 4.3, with coverage factor $k = 2$, that guarantees 95% confidence limits).

$$\Delta(y_E^{(i)}) = k \left(\frac{\max\{y_{Ej}^{(i)}\} - \min\{y_{Ej}^{(i)}\}}{2\sqrt{6}} \right) = \frac{\max\{y_{Ej}^{(i)}\} - \min\{y_{Ej}^{(i)}\}}{\sqrt{6}} \quad (4.2)$$

$\max\{y_{Ej}^{(i)}\}$: maximum among the n concentration values measured in samples exposed in the i^{th} SU;

$\min\{y_{Ej}^{(i)}\}$: minimum among the n concentration values measured in samples exposed in the i^{th} SU.

$$\Delta(y_U) = k \left(\frac{\max\{y_{Uj}\} - \min\{y_{Uj}\}}{2\sqrt{6}} \right) = \frac{\max\{y_{Uj}\} - \min\{y_{Uj}\}}{\sqrt{6}} \quad (4.3)$$

$\max\{y_{Uj}\}$: maximum among the m concentration values measured in unexposed samples;

$\min\{y_{Uj}\}$: minimum among the m concentration values measured in unexposed samples.

Finally, $\Delta(EU^{(i)})$ can be easily calculated according to formula 4.4.

$$\Delta(EU^{(i)}) = EU^{(i)} \sqrt{\left(\frac{\Delta(y_E^{(i)})}{y_E^{(i)}} \right)^2 + \left(\frac{\Delta(y_U)}{y_U} \right)^2} \quad (4.4)$$

Numerical example

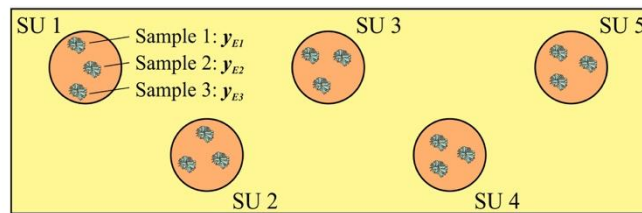
Suppose one wants to assess the bioaccumulation level of chromium (Cr) in *Pseudevernia furfuracea* samples exposed in $N = 5$ SUs in a study area, having $n = 3$ samples exposed for 12 weeks in each SU, and $m = 6$ unexposed samples (Fig. 4.2). For generalization purposes, mean Cr concentrations in the SUs and the unexposed mean value are referred as y_E and y_U , respectively (Fig. 4.2).

Background area



Sample	Concentration ($\mu\text{g g}^{-1}$)
y_{U1}	1.46
y_{U2}	1.32
y_{U3}	1.35
y_{U4}	1.24
y_{U5}	0.86
y_{U6}	0.82
y_U	1.18
$\Delta(y_U)$	0.26

Study area



SU	y_{E1} ($\mu\text{g g}^{-1}$)	y_{E2} ($\mu\text{g g}^{-1}$)	y_{E3} ($\mu\text{g g}^{-1}$)	y_E ($\mu\text{g g}^{-1}$)	$\Delta(y_E)$ ($\mu\text{g g}^{-1}$)
1	1.67	1.90	0.94	1.50	0.39
2	5.50	4.98	4.72	5.07	0.32
3	3.50	3.10	3.10	3.23	0.16
4	4.00	4.00	3.05	3.68	0.39
5	1.80	1.60	1.65	1.68	0.08

Figure 4.2 – Numerical example: initial data.

Concentration values of the element Cr measured in $m = 6$ unexposed samples (y_{Uj}), their mean value and associated uncertainty (y_U and $\Delta(y_U)$), as well as Cr concentration values measured in $n = 3$ samples exposed at each of the $N = 5$ SUs (y_{Ej}), their mean values and associated uncertainties (y_E and $\Delta(y_E)$).

From the exemplifying dataset in Fig. 4.2, the application of formulae 4.1 - 4.4 easily allows to calculate the mean EU ratio, its associated uncertainty, and their difference ($EU - \Delta(EU)$), thus permitting the attribution of bioaccumulation classes to SUs (Table 4.2; Fig. 4.3):

SU	EU	$\Delta(EU)$	$EU - \Delta(EU)$	Class
1	1.27	0.43	0.84	1
2	4.29	0.98	3.31	4
3	2.74	0.62	2.12	3
4	3.12	0.76	2.36	3
5	1.43	0.32	1.10	2

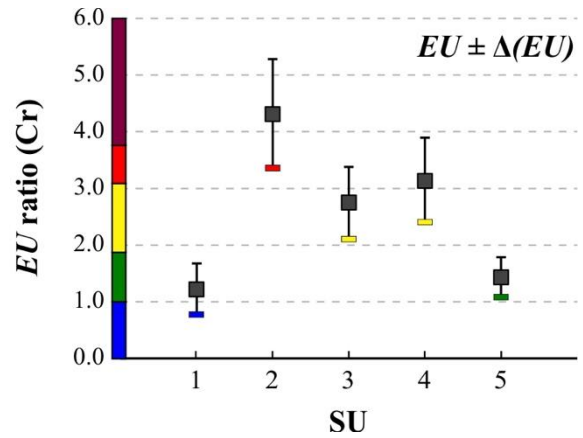


Figure 4.3 – Numerical example: final output

EU ratios and their associated uncertainties for the element Cr in 5 SUs. Their difference, used to assess the class for each SU is also reported, along with a graphic representation of the output.

5. RECOMMENDATIONS FOR QUALITY ASSURANCE AND QUALITY CONTROL

Proper Quality Assurance (QA) and Quality Control (QC) procedures should be implemented to control errors and document the overall quality of the survey/monitoring campaign (see Cline & Burkman, 1989; Shampine, 1993; US EPA, 2002). These procedures form an integral part of the study design and results (see e.g. McCune et al., 1997; Brunialti et al., 2002; Brunialti et al., 2004; Giordani et al., 2009). Studies failing in reporting QA procedures and Quality Control results (with particular reference to point (i) and (ii) reported hereafter) must be regarded as incomplete.

QA and QC procedures should be described in ad-hoc document, termed Quality Assurance Plan (QAP) of the study. The QAP should report the minimum acceptable set of QA/QC procedures. An example of information needed at the end of the survey is reported in Annex E. The QAP should cover the following areas of concern:

- i. Project management. A description of the organization and management of the study and its requirements. This document should include:
 - The identification of responsibilities. Responsible persons of the funding agency and/or of the organization that will carry out the study should be clearly identified as well as their own role and responsibility in the project.
 - The description of the aim of the study, of the problem being targeted and of the final use of the information arising from the study.
 - The sampling objective of the study.
 - the Data Quality Objectives (DQO), i.e., qualitative and quantitative statements that clarifies study's technical and quality objectives. They may vary with the study of concern: they define the appropriate type of data and specify tolerable levels of potential decision errors that will be used as the basis for establishing the quality and quantity of data needed to support decisions.
 - The Data Quality Indicators (DQI): the quantitative statistics and qualitative descriptors used to interpret the degree of acceptability or utility of data. Two indicators could be considered: the Measurement Quality Objective (MQO), defined as the expected level of accuracy of the data (e.g., mean recovery percentages calculated as the percentage ratio between the measured and the expected values for Standard Reference Materials) and the Data Quality Limits (DQL), defined as the minimum acceptable level of consistency between control data and surveyor data.
 - The needs in term of training and expertise of the personnel involved and of any certification and license required.
 - The permission to access private areas or areas with restricted access.
- ii. Standard Operating Procedures (SOP). SOPs should be consistent with the present guidelines and must provide details related to the adaptation needed for the peculiar condition of the study area. If necessary, SOPs must be annexed to the QAP as a separate document. SOPs should be signed by the responsible persons (see first bullet of item (i)) and explicitly accepted by the personnel involved. SOPs should include:
 - The description of the sampling method adopted and its justification in relation to the study objective, including equipment description and field sheets.
 - The description of the method to be used to identify sampling sites location on the maps and in the field.
 - The description of the methods adopted in the field for data and sample collection.
 - The description of the chain of storage of the collected samples.
 - The description of the method of transplant adopted (for lichen transplants method).
 - The description of the chain of custody of the data (recording in the field, storage in electronic format, transmission to central database – if any).
 - The description of software and hardware to be used in the different phases of the study.
- iii. Data Quality Control (QC). The set of activities aimed at ensuring compliance with DQO and DQIs should be described here. QC activity could include:
 - The identification of requirements of the surveying personnel. The certification of the survey team could consider the observer error in each sampling phase.
 - The selection of the sites and trees on the field (for native lichens method).
 - The tree species determination (for native lichens method).
 - The taxonomic identification of the suitable lichen species.

-
- The sample collection and packing.
 - The field exposure of the samples (for lichen transplants method).
 - The sample cleaning and homogenization.
 - The sample storage.
 - The training procedures of the personnel. Description of the training procedures adopted to make personnel familiar with the SOP.
 - The calibration. Description of what exercises will be carried out to promote calibration within the personnel involved.
 - The description of criteria and procedures to be adopted to accept, reject or qualify study information.

ANNEX A. Workflow of standard main phases

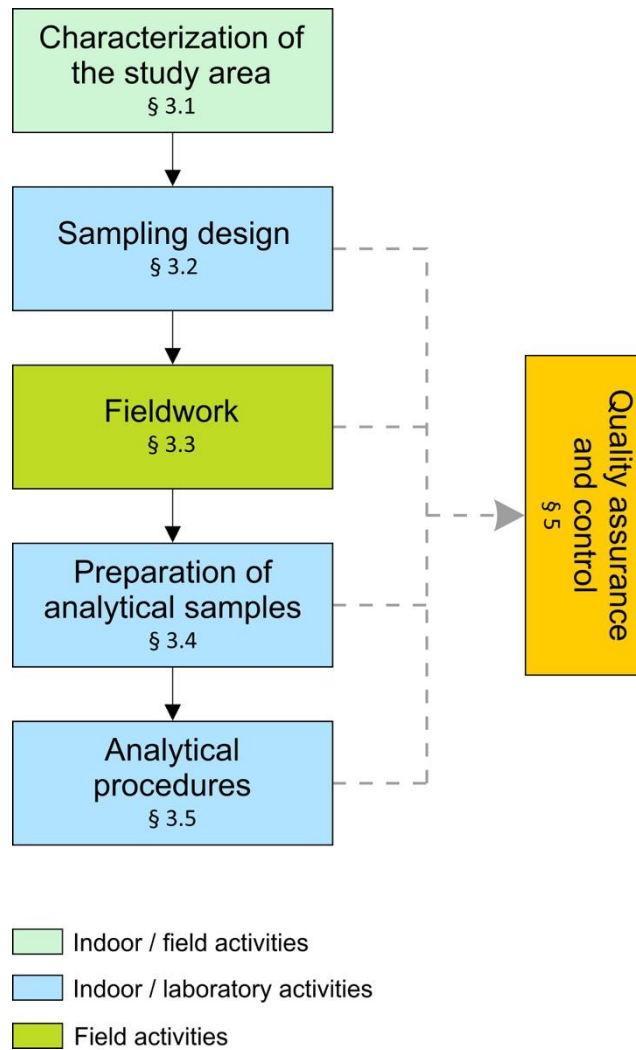


Figure A1 - Workflow illustrating the main phases of a biomonitoring study based on native lichens^N.

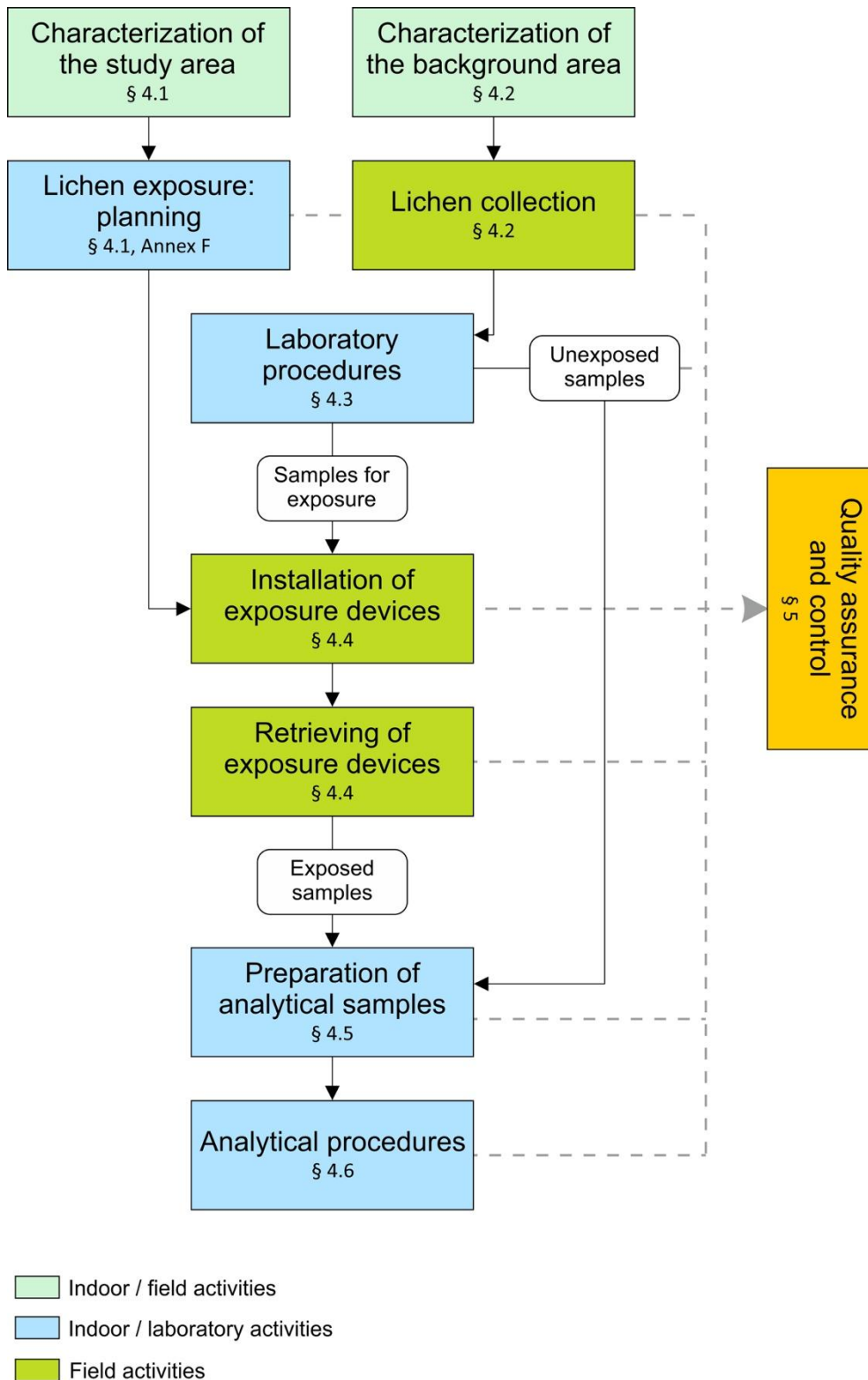


Figure A2 - Workflow illustrating the main phases of a biomonitoring study based on lichen transplants ^T.

ANNEX B. Hints for the identification of suitable lichen species

Relevant characters for the identification of lichen species are the growth form, different types of propagative and reproductive structures, surface structures and colours of the thallus, as well as the results of simple chemical spot tests. Hereafter a list of chemical tests is reported, along with description sheets with the main morphological and ecological features of those species suitable to perform bioaccumulation studies.

Chemical characters of lichens

Lichens produce several secondary metabolites (lichen substances), some of which are effectively used as diacritical characters. Spot test reactions are a quick and inexpensive way to screen for lichen substances. Indeed, several of these compounds typically react with certain chemicals and give colour reactions which assist in the species identification. Some reactions are weak and less persistent so best observed under a stereomicroscope, with the colour observed on absorbent paper placed in contact with the thallus portion involved in the reaction. To perform effective chemical tests, recently prepared solutions should be used on dry tallies. The most common tests are:

- **K (potassium hydroxide).** Many identifications depend on the spot reactions given by potassium (K) in the form of a solution of caustic soda (sodium hydroxide) and water (10% and 90% by volume, respectively).
Positive reaction (K⁺): yellow, orange or red (dirty yellow due merely to clearing effect is a negative reaction).
- **C (sodium hypochlorite).** The C solution is obtained by sodium hypochlorite (bleach) and water (50% each by volume). The solution must be fresh because it deteriorates quickly. Some C reactions are fleeting, and others are not very intense so it is essential to perform the test carefully under a stereomicroscope. In addition, in the use of domestic bleaches, it is necessary to check their composition as they may contain potassium or other ions which can give false positive C reactions.
Positive reaction (C⁺): pink, orange or red, very rarely green.
- **KC.** In this test, potassium hydroxide (K) and sodium hypochlorite (C) must be used sequentially. It is therefore necessary to first apply K, absorb the excess with absorbent paper, immediately after the C in the same thallus portion.
Positive reaction (KC⁺): pink red, rarely violet.
- **Pd (paraphenylenediamine).** Pd is obtained by dissolving some crystals of paraphenylenediamine in 90% ethyl alcohol and using it at the moment. It deteriorates in a few hours. Since paraphenylenediamine is an allergen and is toxic by inhalation and skin contact, the ethanol solution should be handled very carefully. An alternative preparation, more stable and safer to use, is obtained by dissolving 1 g of para-phenylenediamine, 10 g of sodium sulphite and 0.5 ml of detergent in 100 ml of water (Steiner's solution).
Positive reaction (Pd⁺): yellow, orange or red.

For more information about lichen chemical characters refer to:

- <http://www.britishlichensociety.org>.
- Orange et al., 2001.
- Baruffo et al., 2001.

For details about identification of lichen taxa refer to:

- Nimis, 2016.
- Nimis & Martellos, 2017.
- Smith et al., 2009.

Description of suitable species in alphabetical order

Evernia prunastri

Accepted name: *Evernia prunastri* (L.) Ach.

Growth form: fruticose.

Substrata: bark (neutral to acid barked tree trunks).

Photobiont: green algae (*Trebouxia* spp.).

Reproductive strategy: mainly asexual, by soredia.

Distribution: widespread and abundant holarctic lichen with a wide ecological amplitude, rare only in disturbed situations and in dry habitats.

Brief description: thallus (1-)2-6(-10) cm long, 2-4(-6) cm wide, about 1 mm thick, fruticose; lobes rather soft, numerous, strap-shaped, ± palmately branched, often twisted and pendulous; upper surface green-grey to pale green-yellow, often with an incomplete network of elongate ridges spreading towards the margins enclosing hollows; lower surface white, almost lacking photobiont, but occasionally dotted green especially towards the tips; soralia marginal and/or laminal, at first rounded and often confined to eroded surfaces of ridges and/or lobe margins, later becoming confluent, paler or concolorous with the upper surface. Apothecia 2-5 mm diam.

E. prunastri thalli in shaded habitats usually have fewer, paler, often more elongated lobes. Specimens from polluted sites are often reduced in size with contorted or downturned lobes which can be markedly eroded and sorediate. Sometimes they can be confused with *Ramalina farinacea* which, on the contrary, has not a dorsiventral structure.

Medulla C-, K-, KC-, Pd-.

Length of outermost parts of thalli to be selected: 10 - 20 mm.



Figure B1 – *Evernia prunastri* (pictures: <http://dryades.units.it/italic>, CC BY-SA3.0).

Flavoparmelia caperata

Accepted name: *Flavoparmelia caperata* (L.) Hale.

Growth form: foliose, broad lobed.

Substrata: bark of isolated deciduous trees; more rarely on evergreen trees and only exceptionally on rocks.

Photobiont: green algae (*Trebouxia* spp.).

Reproductive strategy: mainly asexual, by soredia.

Distribution: a pantemperate species, common and abundant in the submediterranean belt, mostly along the Tyrrhenian side of the Peninsula, rarer elsewhere; in humid areas common also within eu-Mediterranean vegetation, in arid areas restricted to sheltered situations, e.g. inside open forests.

Brief description: thallus to 20 cm diam., often forming conspicuous, extensive patches, ± closely appressed but becoming somewhat detached towards the centre; lobes 5-13 mm wide, wavy, rounded at apices, ± contiguous at tips but overlapping at the centre; margins often intended; upper surface yellow to yellow-green, occasionally grey-green (in shade), often coarsely corrugate, especially towards the centre, postulate-sorediate; pustules laminal, initially punctiform and intact, eventually coalescing to form more extensive, erose, spreading areas; soredia coarse and granular, occasionally adhering and then forming gnarled lumps; lower surface black, brown towards the lobe margins; rhizines absent from a narrow zone along margin (± 1 mm). Apothecia rare, to 8 mm diam. Sometimes the species can be confused with the relatively common *Flavoparmelia soredians* and the rarer *Flavopunctelia flaventior*.
Cortex K-, medulla C-, K± dirty yellow, KC± red, Pd+ orange-red.

Length of outermost parts of thalli to be selected: 2 - 5 mm.

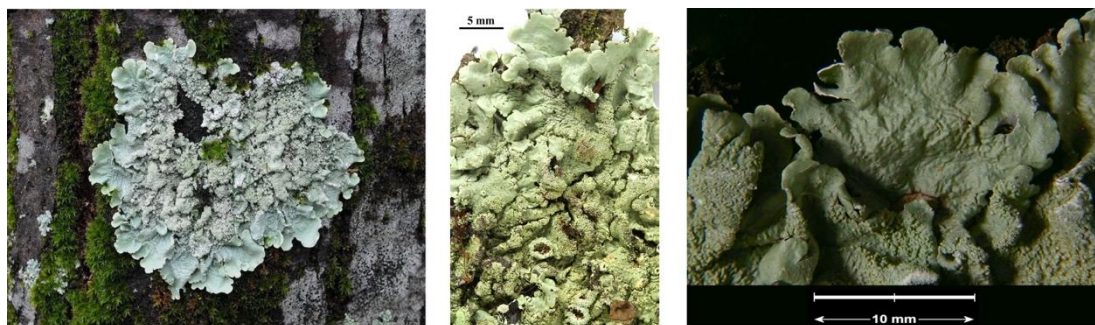


Figure B2 – *Flavoparmelia caperata* (pictures: <http://dryades.units.it/italic>, CC BY-SA3.0).

Hypogymnia physodes

Accepted name: *Hypogymnia physodes* (L.) Nyl.

Growth form: foliose, narrow lobed.

Substrata: bark or wood including conifers and hardwoods, rarely on rock, moss, or alpine sod.

Photobiont: green algae (*Trebouxia* spp.).

Reproductive strategy: mainly asexual, by soredia.

Distribution: a circumpolar, arctic, boreal and montane lichen, from the lowlands to the subalpine belt.

Brief description: thallus to 10 cm diam., ± loosely attached, forming rosettes or irregularly spreading, often forming large patches; lobes 2-3 mm wide, ± hollow, often ascending towards the tips; upper surface grey, smooth ± shining, sorediate; soralia lip-shaped, developing from the rupture of the underside tips of the lobes, becoming upturned and fan-like; lower surface black, pale brown towards margins, wrinkled. Apothecia rather rare, with shorth thick stalks; disc red-brown.

H. physodes is a polymorphic species, ranging from contorted, plate-like thalli with coarse, overlapping and swollen lobes to thin, finely finger-like thalli with discrete lobes. Sometimes the species can be confused with scarcely developed forms of *H. tubulosa*, which has capitate soralia.

Cortex K+ yellow; medulla and soralia C-, K-, KC+ red, Pd+ orange to red.

Length of outermost parts of thalli to be selected: 2 - 5 mm.



Figure B3 – *Hypogymnia physodes* (pictures: <http://dryades.units.it/italic>, CC BY-SA3.0).

Parmelia sulcata

Accepted name: *Parmelia sulcata* Taylor s.lat.

Growth form: foliose, broad lobed.

Substrata: bark (on acid or subacid bark) and firm siliceous rock, exceptionally on wood.

Photobiont: green algae (*Trebouxia* spp.).

Reproductive strategy: mainly asexual, by soredia.

Distribution: pantemperate and southern boreal species, widespread from coastal regions to exposed mountains summits (cosmopolitan, one of the most widely distributed lichens known).

Brief description: Thallus 5-10(-20) cm diam., often forming complete rosettes or randomly intricate, ± loosely attached, lobes to 5 mm wide, apices incised, discrete or contiguous and overlapping, especially at the centre of the thallus; upper surface grey-white to grey-green, sometimes white-pruinose, flat to weakly pitted, with oval or elongate, scattered, white pseudocyphellae that frequently fuse to form a conspicuous, coarse, incomplete network; soralia elongate, laminal and marginal, derived from breakdown to cortex above the pseudocyphellae; soredia granular, eroding; lower surface black, brown towards the margin; rhizinae simple or forked. Apothecia occasional; disc red-brown to dark brown.

P. sulcata s.lat. varies considerably in the size and degree of separation of the lobes, also in the amount of soralia development. *Parmelia saxatilis* differs only in being isidiate and *P. submontana* by the more ascending, strap-like lobes and nature of the soredia.

Cortex K+ yellow → red; medulla and soralia C-, K+ orange, KC+ orange, Pd+ orange.

Length of outermost parts of thalli to be selected: 2 - 5 mm.



Figure B4 – *Parmelia sulcata* (pictures: <http://dryades.units.it/italic>, CC BY-SA3.0).

Pseudevernia furfuracea

Accepted name: *Pseudevernia furfuracea* (L.) Zopf.

Growth form: foliose broad lobed to fruticose.

Substrata: on exposed, well-lit bark and wood, on conifers, mainly on acid-barked deciduous trees, more rarely on siliceous rocks.

Photobiont: green algae (*Trebouxia* spp.).

Reproductive strategy: mainly asexual, by isidia, or isidia-like structures, although apothecia are quite frequent in sub-Alpine populations.

Distribution: cool-temperate to boreal-montane lichen with optimum in the montane and subalpine belts; abundant in the Alps, rarer in the Apennines, exceptionally reaching the plains of northern Italy on very acid substrata.

Brief description: thalli to 10 cm diam., composed of a few to numerous hanging, strap-shaped lobes 1-4 mm wide, dichotomously branched in one plane, branching widely divergent with short side branches; upper surface grey-white, matt, often rough with isidia or small folioles; lower surface usually channelled, uniformly grey-black or \pm mottled black and brownish-white or pinkish, with incurved margins concolorous with upper surface. Apothecia to 1.5(-3) cm diam., lateral, on curved part of branches.

Pseudevernia furfuracea resembles *Evernia prunastri* in habit but it is characterized by the presence of isidia and the naked, channelled, often at least partly blackened lower surface, while *E. prunastri* is only weakly dorsiventral, with a greenish upper surface and a lax medulla, and has a white lower surface.

Cortex K+ yellow; medulla Pd-, C- (*Pseudevernia furfuracea* var. *furfuracea*) or C+ pink to red (*Pseudevernia furfuracea* var. *ceratea*).

Length of outermost parts of thalli to be selected: 15 - 25 mm (e.g., Incerti et al., 2017).



Figure B5 – *Pseudevernia furfuracea* (pictures: <http://dryades.units.it/italic>, CC BY-SA3.0).

Ramalina farinacea

Accepted name: *Ramalina farinacea* (L.) Ach.

Growth form: fruticose.

Substrata: mainly on bark, very rarely on rocks

Photobiont: green algae (*Trebouxia* spp.).

Reproductive strategy: mainly asexual, by soredia.

Distribution: a widespread, Mediterranean-Atlantic to southern boreal lichen found on bark in humid situations, from the mountains to the Mediterranean belt; extinct over much of the northern plains but still common in upland areas.

Brief description: thallus 3-6(-10) cm long, tufted, pendent, arising from a strictly delimited holdfast, often subdivided into numerous flattened branches up to 3 mm wide, yellow- to dark grey-green. Medulla solid, subcortex cartilagineous; soralia numerous, marginal, discrete, circular to ellipsoid, saucer-shaped; soredia 20-30 μm diam., pale yellow-green, farinose. Apothecia rare, lateral.

Ramalina farinacea is chemically and morphologically very polymorphic: may resemble *E. prunastri* but has a tough, cartilaginous thallus, with photobiont cells below all surfaces.

Several chemotypes: (1) medulla and soralia K- or orange-brown, Pd+ orange-red (2) K+ yellow-red, Pd+ yellow-orange (3) K-, Pd-.

Length of outermost parts of thalli to be selected: 15 - 25 mm.



Figure B6 – *Ramalina farinacea* (pictures: <http://dryades.units.it/italic>, CC BY-SA3.0).

Xanthoria parietina

Accepted name: *Xanthoria parietina* (L.) Th. Fr.

Growth form: foliose, broad lobed.

Substrata: on a wide variety of nutrient-rich and -enriched substrata, including tree bark and rocks (wayside trees, inland and coastal calciferous or basic siliceous rocks, roofing tiles, wood or farmyard fences).

Photobiont: green algae (*Trebouxia* spp.).

Reproductive strategy: mainly sexual.

Distribution: cosmopolitan.

Brief description: thallus <15 cm diam., often forming extensive patches, forming ± regular, yellow-orange, but frequently ± grey, appressed, somewhat wrinkled rosettes; lobes ± overlapping, plicate, broadened towards the apices, intended, the apices 1-3 mm wide, rounded or somewhat notched and ± flat; coarse, flat, subsquamulose to ligulate lobules sometimes present towards the centre of the rosette, usually scattered, but occasionally abundant; attached to the substratum by hapters. Apothecia usually numerous, to 4 mm diam., scattered to clustered, sessile to peltate (sometimes ± stalked when on twigs), orbicular to contorted, concave when young, becoming flat when mature, with slightly raised, smooth, concolorous thalline exciple; old apothecia on moribund thalli becoming convex and immarginate.

This common and conspicuous lichen is usually easily identified by its uniformly orange-yellow rosettes with plentiful apothecia of varied age. Grey thalli, with greatly reduced amounts of the yellow pigment parietin are mostly found in shaded habitats. Morphs with lobules are sometimes difficult to distinguish from *X. calcicola* (mostly saxicolous) and *X. aureola*.

Thallus K+ purple.

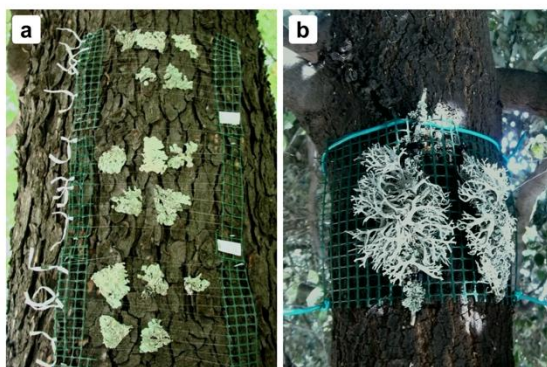
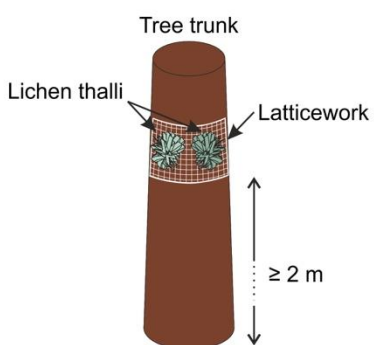
Length of outermost parts of thalli to be selected: 2 - 4 mm (see Fortuna & Tretiach, 2018).



Figure B7 – *Xanthoria parietina* (pictures: <http://dryades.units.it/italic>, CC BY-SA3.0).

ANNEX C. Examples of transplants and exposure devices ^T

Tree trunks



Tree branches

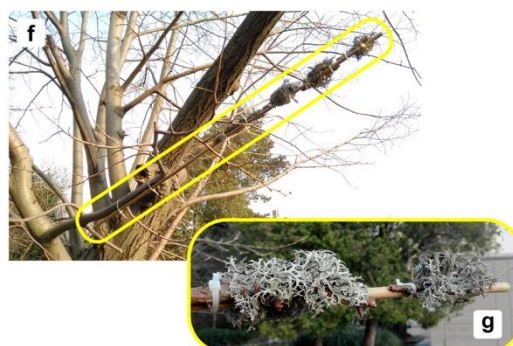
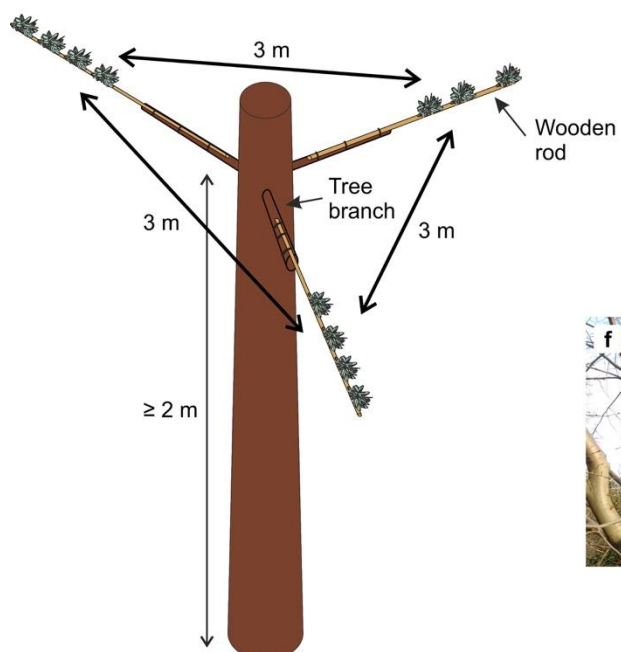
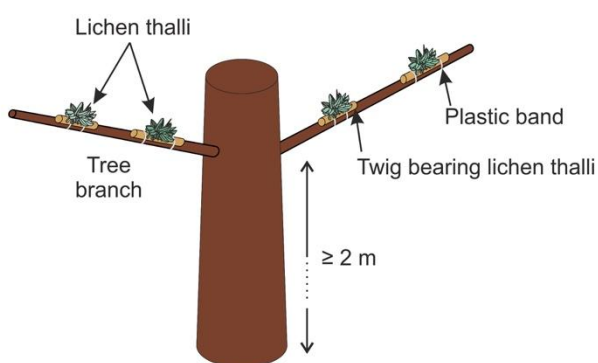


Figure C1 – Example of exposure devices.

Schematic representations and pictures of lichens transplanted on trunks or tree branches. Portions of *Flavoparmelia caperata* thalli on the exposure device (latticework) attached on a tree trunk (a); *Pseudevernia furfuracea* thalli on the exposure device (latticework) attached on a trunk (b); *Evernia prunastri* thalli on tree branches (c); *Ramalina canariensis* thalli on tree branches (d); *P. furfuracea* thalli on exposure devices (wooden rods: c. 0.5 cm diam. and 120 cm long; Kodnik et al., 2015), in turn secured on tree branches (devices are placed at three meters distance among each other) (e); details of *P. furfuracea* thalli on exposure devices (f-g).

ANNEX D. Example of field sheets


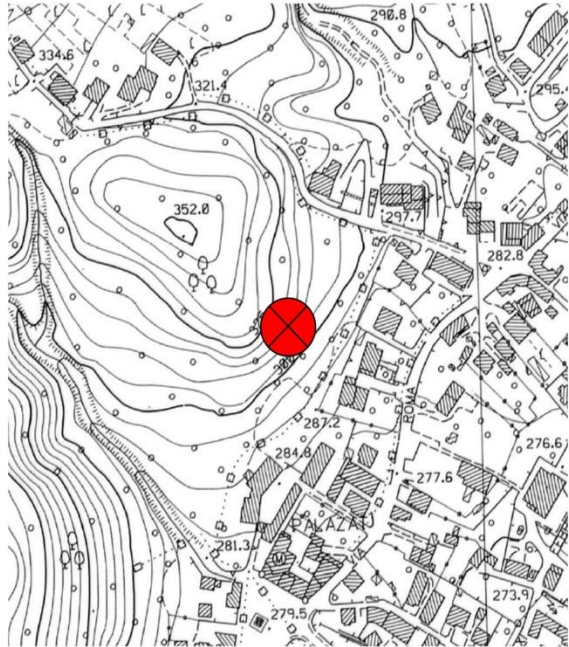
Date	Name/s and institution/s				
SU	Locality				
Latitude (UTM)	Notes				
Longitude (UTM)					
Altitude (m a.s.l.)					
Substrate tree species		Location of the SU			
Standard tree with native lichens (picture)					
					
Mean element concentration values and associated standard deviations ($\mu\text{g g}^{-1}$)					
Al		Cs		S	
As		Fe		Se	
Ba		K		Sn	
Be		Hg		Ti	
Ca		Li		U	
Cd		Ni		V	
Co		Na		Zn	
Cr		Pb		Zr	

Figure D1 – *Biomonitoring by native lichens*^N
 Example of sheet with information on field activities and results obtained for each SU.

Date		Name/s and institution/s
Locality	Locality name	Latitude (UTM)
		Longitude (UTM)
		Altitude (m a.s.l.)
Notes		
Vegetational features	Substrate tree species	
	Notes	
Lithological substrate	Lithological macro-category	<input type="checkbox"/> Limestones and dolomites <input type="checkbox"/> Sandstones <i>sensu lato</i> <input type="checkbox"/> Igneous effusive rocks <input type="checkbox"/> Igneous intrusive rocks <input type="checkbox"/> Metamorphic rocks <input type="checkbox"/> Clastic rocks
	Lithological substrate(s)	
	Presence of outcrops	<input type="checkbox"/> yes <input type="checkbox"/> no
	Notes	
Anthropization	Distance the from nearest built-up areas (km)	
	Presence of nearby rural areas	<input type="checkbox"/> yes (distance: m) <input type="checkbox"/> no
	Presence of nearby road/s	<input type="checkbox"/> yes (distance: m) <input type="checkbox"/> no
	Type of road/s	<input type="checkbox"/> Secondary paved road <input type="checkbox"/> Gravel road <input type="checkbox"/> Other
	Notes	
Additional information		

Figure D2 – *Biomonitoring by lichen transplants*^T.
Example of field sheet for lichen collection in the background area.


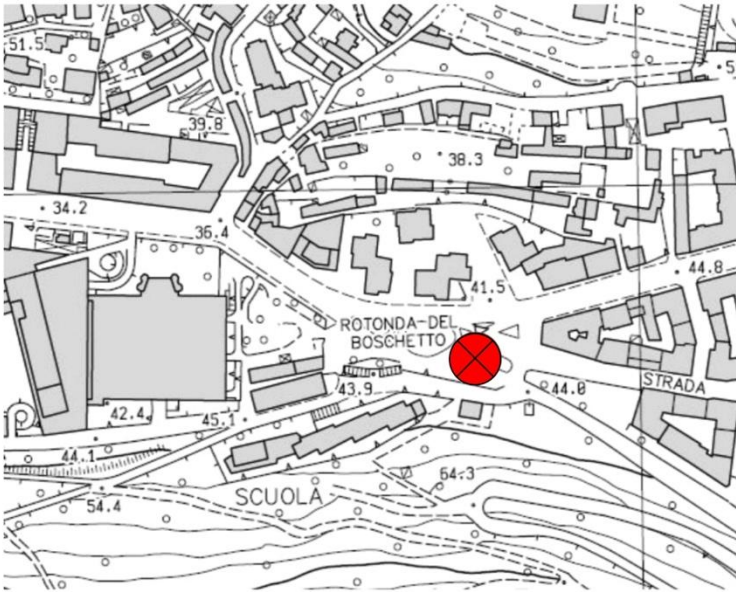
Date	Name/s and institution/s				
SU	Locality				
Latitude (UTM)	Notes				
Longitude (UTM)					
Altitude (m a.s.l.)					
Field support with transplants (picture)	Location of the SU				
					
Mean element concentration values and associated standard deviations ($\mu\text{g g}^{-1}$)					
Al		Cs		S	
As		Fe		Se	
Ba		K		Sn	
Be		Hg		Ti	
Ca		Li		U	
Cd		Ni		V	
Co		Na		Zn	
Cr		Pb		Zr	

Figure D3 – Biomonitoring by lichen transplants ^T.
 Example of sheet with information on field activities and results obtained for each SU.

ANNEX E. Information needed at the end of the biomonitoring survey

Each study should present the following basic data in a table:

- Project management.
- Adopted Standard Operating Procedures.
- Project manager(s).
- Name of the operator(s);
- Temporal framework of the survey.
- Geographical name of the background area ^T.
- Geographical name of the study area.
- Selected lichen species.
- Substrate tree species in the background area ^T or in the study area ^N.
- Part of the tree selected for the lichen exposure (trunk or branches) and their location with respect to the tree crown (under the tree crown, outside the tree crown) ^T.
- Number and size of the sampling units.
- List of target chemical elements.
- Standard deviation of the response variable(s) in the SUs.
- Mean recovery percentages and 95% confidence limits for each element certified in a lichen SRM.
- Data Quality Control manager(s).
- Data Quality Control activity results.

ANNEX F. Probabilistic sampling methods

To achieve representative sampling, SUs have to be located according to an appropriate sampling plan, meant to minimize the times and costs without sacrificing relevant statistical information. Any probability-based sampling strategy for the exposure of lichen samples in the study domain (sect. 2.2) is allowed. The most used sampling approaches are systematic, simple random, stratified random and cluster sampling; other and more complex methods can be easily found in the literature (e.g., Manly & Alberto, 2014. Introduction to ecological sampling. CRC Press). Irrespective the selection of a specific sampling strategy, its description must always be included in the final technical report.

Systematic Sampling

Systematic Sampling (SS) is a type of probability sampling method in which SUs are selected in the study domain according to a random starting point and a fixed spatial interval (Fig. F1). This interval (the sampling interval), is calculated by dividing the study area by the desired sample size (sampling interval is determined beforehand and the starting point is randomly selected). In the example below, the study domain is subdivided into quadrats of 1 km² labelled with alphanumeric codes, and SUs are systematically placed at the centre of each quadrat.

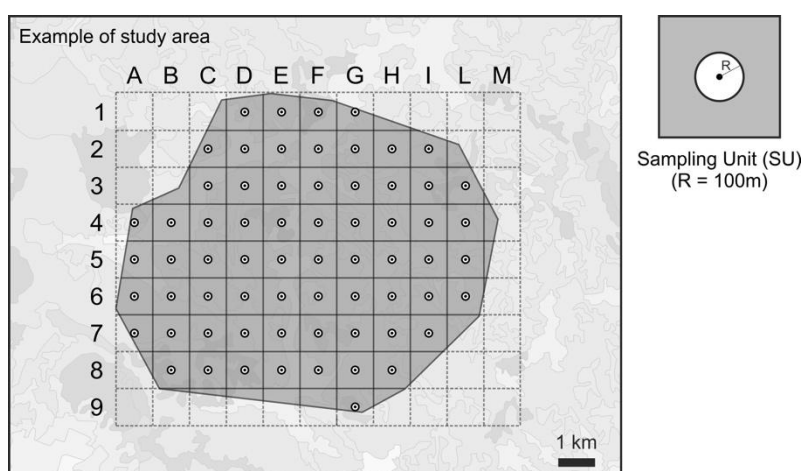


Figure F1 – Systematic sampling.

A special case of SS consists in placing SUs at fixed intervals along linear transects (Fig. F2). This sampling strategy may be suitable when pollutant deposition gradients are highlighted in pilot studies. Moreover, linear transects may be placed according to the direction of prevailing winds in case of point sources of pollution (e.g., Tretiach et al., 2011; Nannoni et al., 2017).

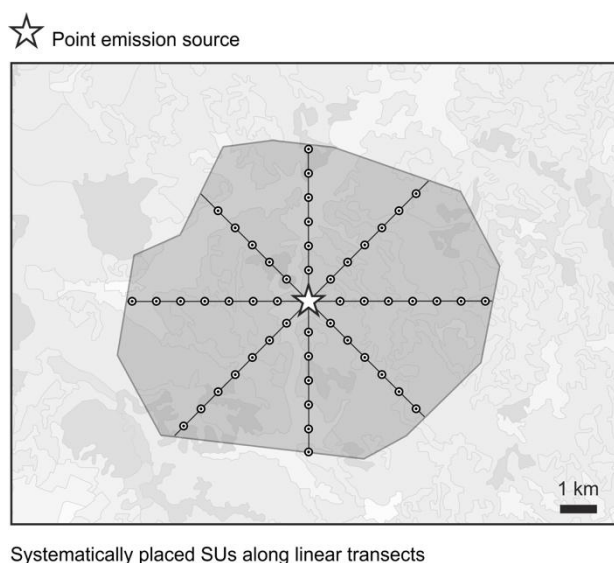


Figure F2 – *Systematic sampling along linear transects.*

Random Sampling

In simple Random Sampling (RS), each SU is randomly located in the study domain by randomly selecting (i) the coordinates of its centre (Fig. F3-a) or (ii) a quadrat within the study area (Fig. F3-b); in the latter case, the SU is systematically placed in the centre of the selected quadrat. This sampling strategy is particularly suitable for homogeneous areas of limited spatial extent. However, in large and environmentally heterogeneous study areas, the RS may significantly increase costs related to travel between SUs.

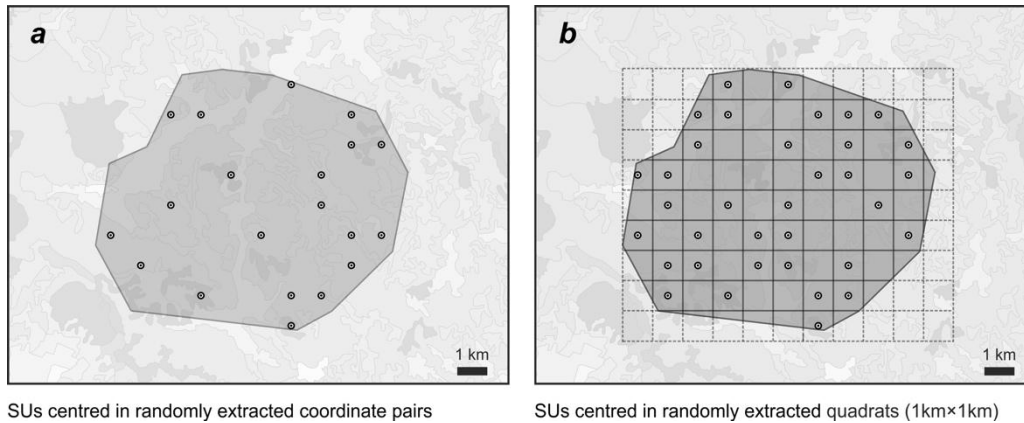


Figure F3 – *Random sampling.*

A special case of RS is the Stratified Random Sampling (SRS), a suitable strategy in case of heterogeneous study areas. For instance, this approach can be successfully used in case of study areas heterogeneous in terms of land use, and in that case, different *strata* (sect. 2.2) must be defined for different land uses. Within each *stratum*, SUs can be selected based on either the random extraction of (i) centre coordinates (Fig. F4-a) or (ii) quadrats within the study area (Fig. F4-b).

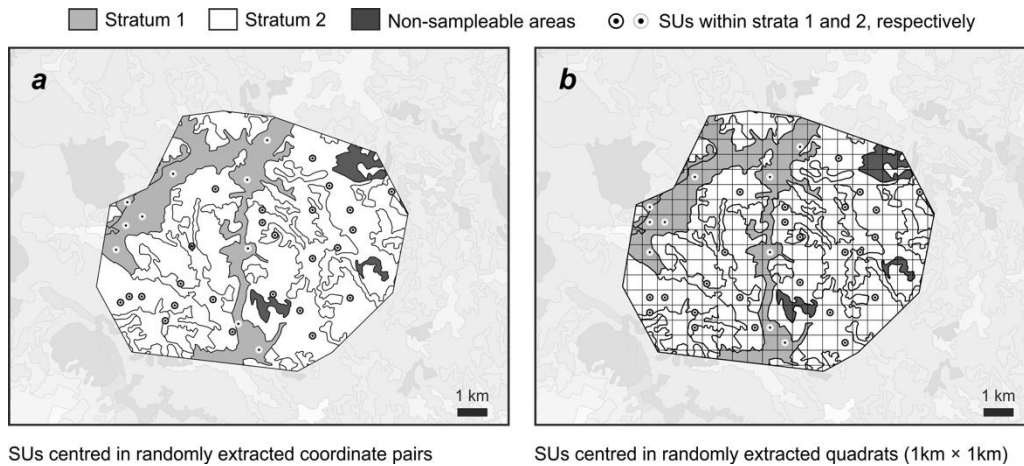
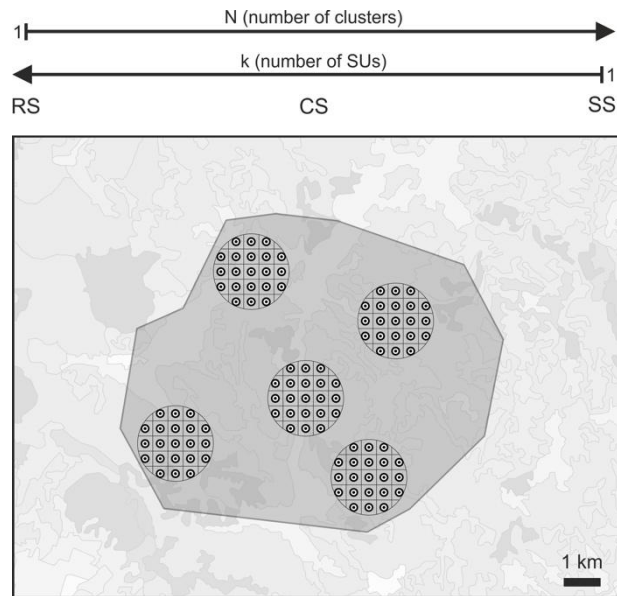


Figure F4 – *Stratified random sampling*

Cluster Sampling

The Cluster Sampling (CS) can be considered a trade-off strategy between RS and SS (Fig. F5). In CS, a certain number of points is randomly selected within the study domain, thus a set of SUs is systematically placed around them (SU clusters). Similarly, SUs may be systematically placed within randomly placed areas of a certain extent, in turn subdivided in sub-areas (e.g., in Fig. F5, SUs are placed within circular areas subdivided into quadrats). The CS solves some typical problems of the simple RS. In particular, when the sampling domain is very large, this strategy allows a higher sampling density; on the other hand, the results of within-cluster SUs may suffer of spatial autocorrelation; moreover, the final sample size will be given by the number of clusters and not by the overall number of SUs.



Systematically placed Sus within randomly placed clusters

Figure F5 – Cluster sampling.

Exclusion/inclusion criteria for edge SUs

Exclusion/inclusion criteria for SUs tangent or secant the edges of the study domain should be defined contextually to the sampling planning. A simple approach is to include only those SUs with a certain surface percentage included in the study domain (e.g. > 50%; Fig. F6).

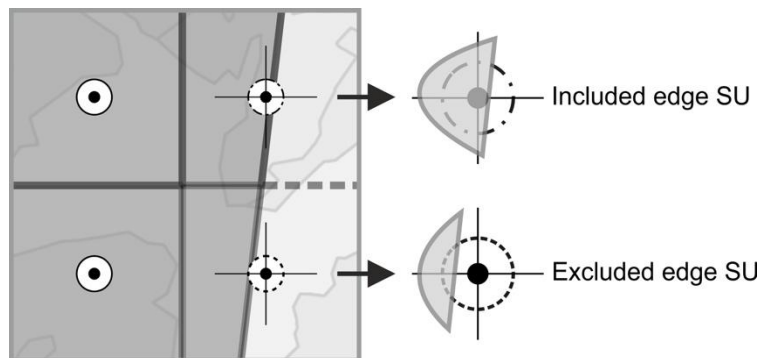


Figure F6 – A possible exclusion/inclusion criteria of edge SUs.

ANNEX G. Preliminary assessment of the lichen elemental content variability in a potential background area^T

Premise

In order to enhance the quality of bioaccumulation data, the variability of the content of target elements in the source lichen population must be assessed before proceeding with the standard actions of a transplant-based biomonitoring study. Therefore, a pilot study is mandatory when a collection area is selected for the first time. In particular, the operator(s) must collect the target species in a remote area that has been fully characterized (sect. 4.2.2); even the lichen collection and the sample preparation must follow these guidelines (sects.4.2, 4.3, 4.5).

Hereafter, a method based on a resampling procedure of bioaccumulation data from a background area is proposed with the aim of assessing the element content variability of the source lichen population. Such a variability is assessed in terms of the confidence limits of the target estimators, i.e. the mean element concentration values in unexposed samples (see definition in sect. 2.2). By implementing the method, the operator(s) shall be able to assess the minimum number of unexposed samples to be analysed in subsequent biomonitoring applications relying on the selected source population.

Note. The reliability of the method is strongly related to the sample size, therefore at least 30 samples must be collected and analysed to this purpose.

Method implementation: R script

The method is illustrated using a working dataset of iron (Fe) concentrations ($\mu\text{g g}^{-1}$ DW) measured in $N = 41$ samples of *Pseudevernia furfuracea* collected in a widely acknowledged background area of the eastern Italian Alps (Cecconi et al., 2018). Preliminarily, the working dataset was screened for upper outliers according to the Tukey method.

The resampling procedure proposed hereafter is based on a script runnable in R environment with packages ‘dplyr’ and ‘reshape’. The script allows to perform a random selection with replacement of $3 \leq n \leq N-1$ samples of *P. furfuracea* (in the script, N is ‘length(Fe)’, i.e. the length of the column vector ‘Fe’). The random selection is iterated 1000 folds for each resampled sub-dataset of dimension ‘ n ’ (henceforth, subset dimension). For each iteration, the mean Fe concentration value is calculated and then, for each subset dimension n , such mean values are furtherly averaged to obtain overall mean values (in the script, ‘Mean’). Similarly, variances are calculated for each iteration, and then averaged within each subset dimension n to obtain overall mean variances (‘S2’). Moreover, standard deviations (‘Sd’), standard errors (‘Se’), coefficients of variation (‘CV’), upper and lower 95% confidence limits (‘uCI’ and ‘lCI’), confidence intervals (‘CI95’), and uncertainties (‘Unc’; calculated as the ratio between the 95% confidence interval and the mean) are calculated from the overall means and the overall mean variances for each n .

All descriptive statistics listed above are saved in R environment in a matrix named ‘Table’ (Table G1). Finally, the script allows to plot two graphs: (i) the overall mean Fe content \pm CI95 vs. the subset dimension n (Fig. G1-a), and (ii) the uncertainty vs. the subset dimension n (Fig. G1-b).

Note. The same R script has to be run for each target element.

```
Fe<-c(322,496,366,348,319,231,239,326,400,300,300,300,300,300,300,300,300,300,300,300,300,237,218,398,365,243,237,241,199,357,366,168,130,140,150,140,140,160,170,160,180,180,180,220,250)
```

```
library(dplyr)
# Hadley Wickham, Romain François, Lionel Henry and Kirill Müller (2018).
# dplyr: A Grammar of Data Manipulation. R package version 0.7.6.
# https://CRAN.R-project.org/package=dplyr
```

```
library(reshape)
# H. Wickham. Reshaping data with the reshape package. Journal of
# Statistical Software, 21(12), 2007.
```

```
M<-matrix(ncol=length(Fe)-1, nrow=1000*(length(Fe)-3))
for (i in 3:(length(Fe)-1))
{
```

```

for (j in 1:1000)
{
  B<-sample(Fe, i, replace=TRUE)
  c<-(length(Fe)-1)-i
  B<-c(B, rep(NA,c))
  as.vector(B)
  ifelse(i==3, x<-j, x<-(j+(1000*i)-3000))
  M[x, ]<-B
}
}

Value<-rowMeans(M, na.rm=TRUE)
n<-length(Fe)-1-(apply(M, 1, function(z) sum(is.na(z))))
Var<-apply(M, 1, var, na.rm=TRUE)
M_mean<-as.data.frame(cbind(n, Value))
M_var<-as.data.frame(cbind(n, Var))
n_cat<-as.data.frame(M_mean$n)
Mean<- aggregate(x = M_mean, by = n_cat, FUN = mean)
S2<- aggregate(x = M_var, by = n_cat, FUN = mean)

Table<-as.data.frame(cbind(Mean$n, Mean$Value, S2$Var))
colnames(Table)<-c("n", "Mean", "S2")

Table$Sd<-sqrt(Table$S2)
Table$Se<-Table$Sd/sqrt(Table$n)
Table$CV<-Table$Sd/Table$Mean
Table$uCI<-Table$Mean+qt(1-0.05/2, Table$n-1)*Table$Sd/sqrt(Table$n)
Table$lCI<-Table$Mean-qt(1-0.05/2, Table$n-1)*Table$Sd/sqrt(Table$n)
Table$CI95<-(qt(1-0.05/2, Table$n-1))*Table$Sd/sqrt(Table$n)
Table$Unc<-((Table$CI95)/Table$Mean)*100

Table

write.table(Table, file="Fe.txt", quote=T, sep=" ", dec=".", na="NA",
row.names=T, col.names=T)

plot(Table$n, Table$Mean, ylim=range(c(0, Table$Mean+Table$CI95)))
arrows(Table$n, Table$Mean-Table$CI95, Table$n, Table$Mean+Table$CI95,
length=0.05, angle=90, code=3)
abline(h=10, col="red")

plot(Table$n, Table$Unc)
abline(h=20, col='blue')

```

Table G1 - Main output of the R script.

Modified output table obtained by the R script. Data refer to the subset dimension (n), overall mean (Mean) and overall mean variance (S2) of Fe concentration, as well as to the corresponding standard deviation (Sd), standard error (Se), coefficient of variation (CV), upper and lower 95% confidence limits (uCI and lCI), confidence interval (CI95), and associated percent uncertainty (Unc).

n	Mean	S2	Sd	Se	CV(%)	uCI	lCI	CI95	Unc(%)	n	Mean	S2	Sd	Se	CV(%)	uCI	lCI	CI95	Unc(%)
3	260	8034	90	52	34.5	483	37	223	85.7	22	258	7823	88	19	34.2	298	219	39	15.2
4	256	7735	88	44	34.3	396	116	140	54.6	23	258	7770	88	18	34.1	296	220	38	14.8
5	257	7949	89	40	34.7	368	146	111	43.1	24	259	7852	89	18	34.3	296	221	37	14.5
6	258	7958	89	36	34.6	351	164	94	36.3	25	258	7852	89	18	34.3	295	222	37	14.2
7	257	7791	88	33	34.3	339	176	82	31.7	26	258	7671	88	17	33.9	293	223	35	13.7
8	259	7922	89	31	34.4	333	185	74	28.7	27	257	7833	89	17	34.4	292	222	35	13.6
9	258	7812	88	29	34.2	326	190	68	26.3	28	259	7925	89	17	34.4	294	224	35	13.3
10	258	8066	90	28	34.8	322	194	64	24.9	29	257	7829	88	16	34.4	291	224	34	13.1
11	258	7720	88	26	34.1	317	199	59	22.9	30	259	7788	88	16	34.1	292	226	33	12.7
12	258	7870	89	26	34.4	314	202	56	21.9	31	258	7841	89	16	34.3	291	226	32	12.6
13	258	7737	88	24	34.1	311	205	53	20.6	32	258	7907	89	16	34.4	290	226	32	12.4
14	260	8040	90	24	34.5	311	208	52	19.9	33	258	7976	89	16	34.6	290	227	32	12.3
15	258	7850	89	23	34.3	307	209	49	19.0	34	258	7878	89	15	34.3	289	227	31	12.0
16	258	7934	89	22	34.5	306	211	47	18.4	35	259	7832	88	15	34.2	290	229	30	11.7
17	258	7821	88	21	34.2	304	213	45	17.6	36	258	7897	89	15	34.5	288	228	30	11.7
18	258	7862	89	21	34.4	302	214	44	17.1	37	258	7850	89	15	34.3	288	229	30	11.4
19	258	7746	88	20	34.1	300	215	42	16.5	38	259	7852	89	14	34.2	288	230	29	11.2
20	258	7592	87	19	33.8	298	217	41	15.8	39	258	7800	88	14	34.3	286	229	29	11.1
21	258	7870	89	19	34.4	298	218	40	15.7	40	258	7875	89	14	34.4	286	229	28	11.0

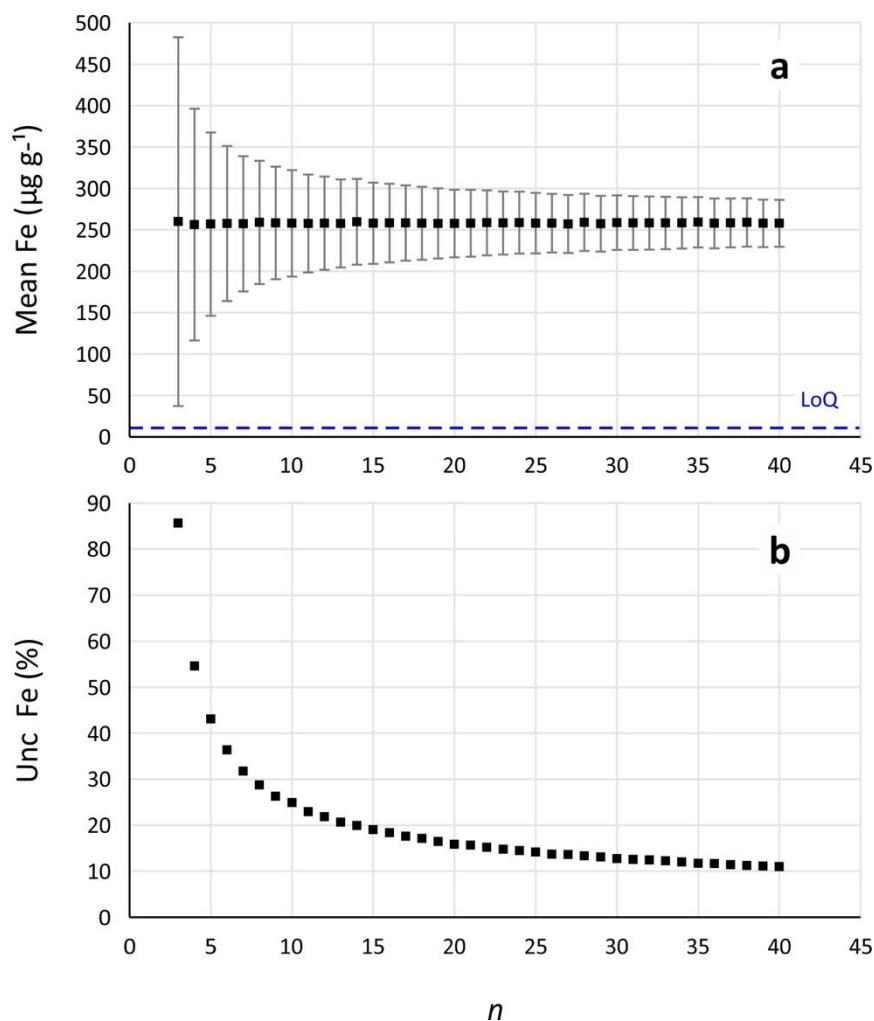


Figure G1 – Graphical output of the R script.

Modified graphical output of the R script. Overall mean Fe concentrations (Mean Fe) vs. subset dimension n (error bars indicate 95% confidence intervals), with indication of the Limit of Quantification (LoQ, blue dashed line); for each targeted element, the LoQ of the analytical procedure should always be lower than the overall mean value corresponding to the smallest subset dimension ($n = 3$) diminished by the 95% confidence interval: $LoQ < Mean - CI95$ (a). Percent uncertainty associated to Fe (Unc Fe) vs. subset dimension n (b).

Assessment of the number of unexposed samples to analyse

The minimum number of unexposed samples to be subjected to analytical determination (N^*) in subsequent transplant-based biomonitoring studies will be given by the arithmetic mean of the subset dimensions corresponding to a percent uncertainty of c. 20% (n^*) calculated for each target element, as shown in Fig. G2 for the exemplifying element Fe.

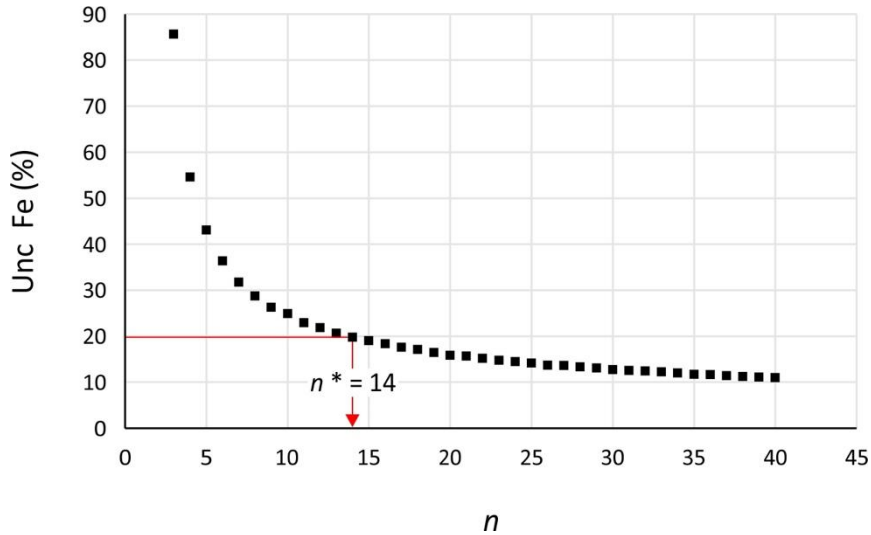


Figure G2 - Subset dimension corresponding to 20% uncertainty.

Subset dimension corresponding to a percent uncertainty of c. 20% (n^*) for the exemplifying element Fe. The uncertainty corresponding to $n^* = 14$ is 19.9% (see Table G1).

On grounds of practical feasibility, and to avoid the use of lichen material affected by an excessive element content variability, the number of unexposed samples to be analysed (N^*) should not exceed 25. If not, the selected area could not be considered a background area.

Let's consider a set of m target elements, and let n_i^* be the subset dimension of the i^{th} element corresponding to c. 20% uncertainty. The minimum number of unexposed samples to analyse will be given by:

$$N^* = \frac{\sum_{i=1}^m n_i^*}{m} \leq 25$$

Once assessed, such a number can be kept constant for subsequent biomonitoring campaigns relying on the same source lichen material.

ANNEX H. Mapping procedures

The results of biomonitoring surveys based on both native lichens (chapter 3) and lichen transplants (chapter 4) must be summarised by means of intelligible cartographic elaborations aimed at reporting the concentrations of bioaccumulated elements in each SU and their spatial patterns. Cartographic elaborations can be easily realized with mapping software (e.g. Arcgis®, Qgis, R, etc.) using as input datasets the matrices of element concentrations or B and EU ratios (sect. 3.7.2 and 4.8.2) in the SUs.

Typically, two cartographic approaches are suitable for biomonitoring data: the first is descriptive and meant to represent the bioaccumulation classes and their associated uncertainties (i.e., descriptive approach), whereas the second approach implies geostatistical interpolation of element concentrations or B and EU ratios in order to spatially represent pollutant patterns in the study area (i.e., geostatistical approach).

Descriptive approach

These cartographic elaborations represent SUs on the basis of their bioaccumulation class and the associated relative uncertainty (RU). It is worth to consider that the calculation and the mapping of the relative uncertainty guarantee the possibility to perform inter-study comparison. In particular, for the i^{th} SU, the relative uncertainty can be calculated as follows:

$$\text{Native lichens: } RU^{(i)} = \frac{\Delta(B^{(i)})}{B^{(i)}} \times 100; \text{ Lichen transplants: } RU^{(i)} = \frac{\Delta(EU^{(i)})}{EU^{(i)}} \times 100$$

$B^{(i)}, EU^{(i)}$: mean values of B and EU ratios for the i^{th} SU (formula 3.1, sect. 3.6; formula 4.1, sect. 4.7);

$\Delta(B^{(i)}), \Delta(EU^{(i)})$: uncertainties of $B^{(i)}$ and $EU^{(i)}$ (formula 3.3, sect 3.7.3; formula 4.4 sect. 4.8.3).

In these maps, colours and sizes of symbols representing SUs have to be chosen according to the corresponding bioaccumulation classes (Tables 3.3 and 4.2 in sects. 3.7.2 and 4.8.2) and their relative uncertainties. An example of cartographic output for lichen transplants is provided in Fig. H1.

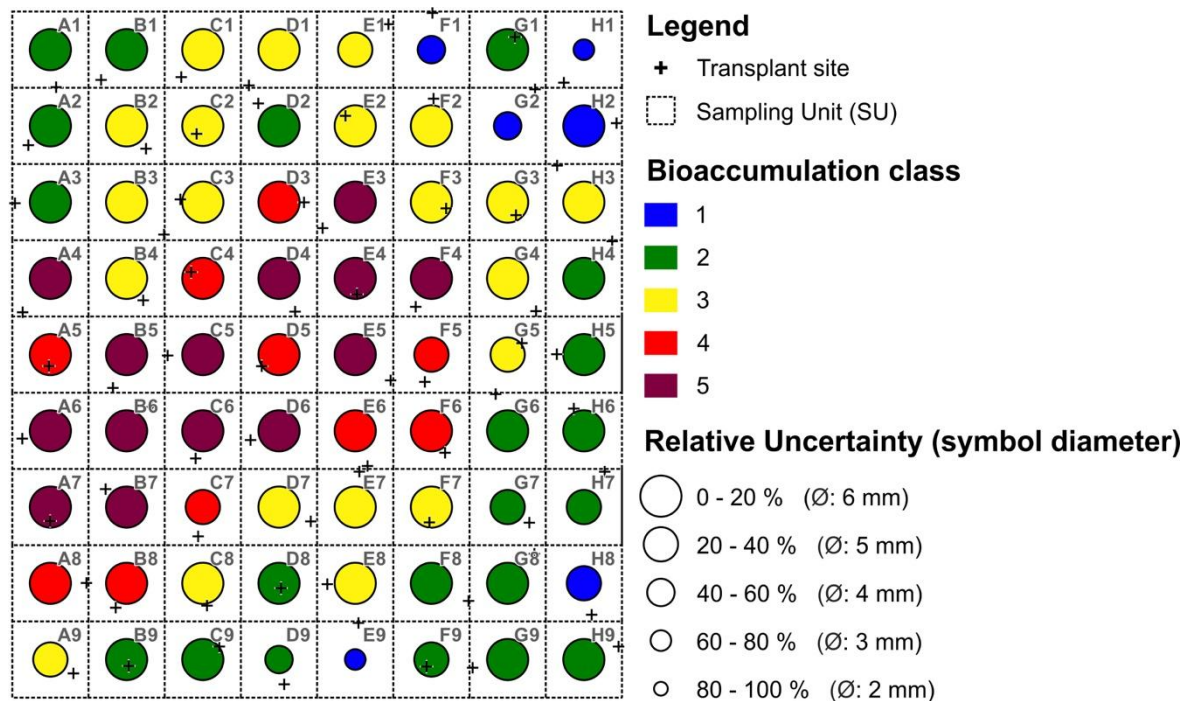


Figure H1 – Example of descriptive representation of SUs (lichen transplant).

Bioaccumulation classes and associated uncertainties are shown by different colours (see Table 4.2) and symbol sizes for each SU in the study area.

Geostatistical approach

In order to recognize spatial patterns of target pollutants, the *EU* and *B* ratios, or alternatively the element concentration values (the target variables), have to be subjected to geostatistical interpolation. In this context, interpolation methods based on non-deterministic algorithms, such as Kriging, are highly recommended. Indeed, Kriging algorithms, besides predicting the values of the target variable at any point of the study area (Fig. H2-a), also provide an estimate of the uncertainty of the interpolated surfaces (Fig. H2-b), depending on statistical model used (e.g., the so-called semi-variogram; Tao, 1995). For a complete description of interpolation methods and their field of applicability, see the online manual of cited software packages and, for instance, Aboal et al. (2006) and Real et al. (2003).

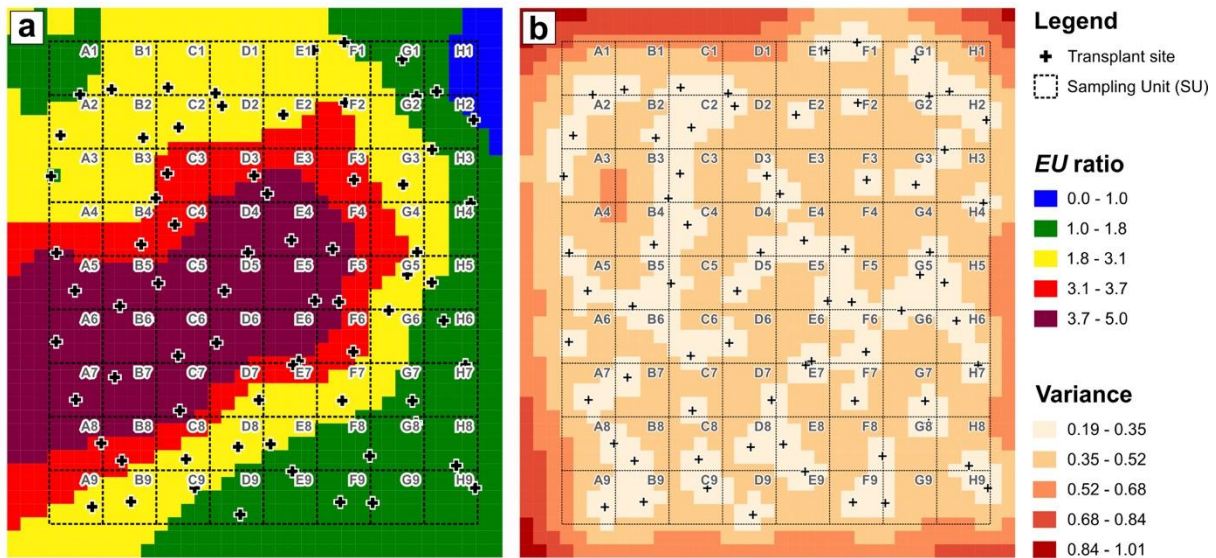


Figure H2 - Example of geostatistical interpolation on *EU* ratio data (lichen transplants).

Spatial pattern of *EU* ratio obtained with an ordinary Kriging interpolation method (a); uncertainty of the interpolation surface expressed as variance (b).

REFERENCES

- Aboal JR, Real C, Fernández JA, Carballeira A (2006) Mapping the results of extensive surveys: the case of atmospheric biomonitoring and terrestrial mosses. *Science of the Total Environment* 356, 256-274.
- Adamo P, Giordano S, Vingiani S, Castaldo Cobianchi R, Violante P (2003) Trace element accumulation by moss and lichen exposed in bags in the city of Naples (Italy). *Environmental Pollution* 122, 91-103.
- Adamo P, Bargagli R, Giordano S, Modenesi P, Monaci F, Pittao E, Spagnuolo V, Tretiach M (2008) Natural and pre-treatments induced variability in the chemical composition and morphology of lichens and mosses selected for active monitoring of airborne elements. *Environmental Pollution* 152(1), 11-19.
- Bargagli R, Mikhailova I (2002) Bioaccumulation of inorganic contaminants. In: Nimis PL, Scheidegger C, Wolseley PA (Eds.), *Monitoring with Lichens-Monitoring Lichens*. Springer-Verlag, Berlin, 324 pp.
- Bargagli R, Losco FP, D'Amato ML (1987) Zonation of trace metal accumulation in three species of epiphytic lichens belonging to the genus *Parmelia*. *Cryptogamie, Bryologie, Lichénologie* 8, 331-337.
- Baruffo L, Tretiach M, Zedda L, Leuckert Ch (2001) Sostanze licheniche: come riconoscerle e perché. *Notiziario della Società Lichenologica Italiana* 14, 5-33.
- Bettinelli M, Spezia S, Bizzarri G (1996) Trace elements determination in lichens by ICP-MS. *Atomic Spectroscopy* 17, 133-141.
- Bossard M, Feranec J, Otahel J (2000) CORINE land cover technical guide – Addendum 2000. European Environment Agency. EEA Copenhagen.
- Brunialti G, Frati L (2007) Biomonitoring of nine elements by the lichen *Xanthoria parietina* in Adriatic Italy: a retrospective study over a 7-year time span. *Science of the Total Environment* 387, 289-300.
- Brunialti G, Giordani P, Isocrono D, Loppi S (2002) The evaluation of data quality in lichen biomonitoring studies: the Italian experience. *Environmental Monitoring and Assessment* 75, 271-280.
- Brunialti G, Giordani P, Ferretti M (2004) Discriminating between the good and the bad: quality assurance is central in biomonitoring studies. *Environmental Monitoring* 20, 443-464.
- Cecconi E, Incerti G, Capozzi F, Adamo P, Bargagli R, Benesperi R, Candotto Carniel F, Favero-Longo SE, Giordano S, Puntillo D, Ravera S, Spagnuolo V, Tretiach M (2018) Background element content of the lichen *Pseudevernia furfuracea*: A supra-national state of art implemented by novel field data from Italy. *Science of the Total Environment* 622, 282-292.
- Cecconi E, Fortuna L, Benesperi R, Bianchi E, Brunialti G, Contardo T, Di Nuzzo L, Frati L, Monaci F, Munzi S, Nascimbene J, Paoli L, Ravera S, Vannini A, Giordani P, Loppi S, Tretiach M (2019a) New interpretative scales for lichen bioaccumulation data: the Italian proposal. *Atmosphere* 10, 136.
- Cecconi E, Incerti G, Capozzi F, Adamo P, Bargagli R, Benesperi R, Candotto Carniel F, Favero-Longo SE, Giordano S, Puntillo D, Ravera S, Spagnuolo V, Tretiach M (2019b). Background element content in the lichen *Pseudevernia furfuracea*: A comparative analysis of digestion methods. *Environmental Monitoring and Assessment* 191, 260.
- Cline SP, Burkman WG (1989) The role of quality assurance in ecological programs. In: Bucher JB, Bucher-Wallin I (Eds.), *Air Pollution and Forest Decline, Proceedings of the 14th International Meeting for Specialists on Air Pollution Effects on Forest Ecosystems*, IUFRO P2.05, Inter-laken, Switzerland, Oct. 2-8 (1988) Birmensdorf, pp. 361-365.
- Elzinga CL, Salzer DW, Willoughby JW, Gibbs JP (2001) *Monitoring plant and animal populations*. Blackwell Science, Malden, Massachusetts.

-
- Fortuna L, Tretiach M (2018) Effects of site-specific climatic conditions on the radial growth of the lichen biomonitor *Xanthoria parietina*. *Environmental Science and Pollution Research* 25(34), 34017-34026.
- Fрати L, Brunialti G, Loppi S (2005) Problems related to lichen transplants to monitor trace element deposition in repeated surveys: a case study from Central Italy. *Journal of Atmospheric Chemistry* 52, 221-230.
- Giordani P, Brunialti G, Benesperi R, Rizzi G, Frati L, Modenesi P (2009) Rapid biodiversity assessment in lichen biomonitoring surveys: implications for quality assurance. *Journal of Environmental Monitoring* 11, 730-735.
- Hale ME, Lawrey JD (1985) Annual rate of lead accumulation in the lichen *Pseudoparmelia baltimorensis*. *The Bryologist* 88, 5-7.
- Honegger R (2003) The impact of different long-term storage conditions on the viability of lichen-forming ascomycetes and their green algal photobiont, *Trebouxia* spp. *Plant Biology* 5, 324-330.
- Incerti G, Cecconi E, Capozzi F, Adamo P, Bargagli R, Benesperi R, Candotto Carniel F, Cristofolini F, Giordano S, Puntillo D, Spagnuolo V, Tretiach M (2017) Intraspecific variability in baseline element composition of the epiphytic lichen *Pseudevernia furfuracea* in remote areas: implications for biomonitoring of air pollution. *Environmental Science and Pollution Research* 24, 8004-8016.
- Jensen M (2002) Measurement of chlorophyll fluorescence in lichens. In: Kranner IC, Beckett RP, Varma AK (Eds.) *Protocols in Lichenology*. Springer Lab Manuals. Springer, Berlin, Heidelberg, pp. 135-151.
- Kodnik D, Candotto Carniel F, Lichen S, Tolloi A, Barbieri P, Tretiach M (2015) Seasonal variations of PAHs content and distribution patterns in a mixed land use area: a case study in NE Italy with the transplanted lichen *Pseudevernia furfuracea*. *Atmospheric Environment* 113, 255-263.
- Loppi S, Ravera S, Paoli L (2019) Coping with uncertainty in the assessment of atmospheric pollution with lichen transplants. *Environmental Forensics*, DOI: 10.1080/15275922.2019.1627615.
- Manly BF, Alberto JAN (Eds.) (2014) *Introduction to ecological sampling*. CRC Press.
- Markert B (1995) Sample preparation (cleaning, drying, homogenization) for trace element analysis in plant matrices. *Science of the Total Environment* 176, 45-61.
- McCune B, Dey J, Peck J, Cassell D, Heiman K, Will-Wolf S, Neitlich P (1997) Repeatability of community data: species richness versus gradient scores in large-scale lichen studies. *The Bryologist* 100, 40-46.
- Nannoni F, Mazzeo R, Santolini R., Protano G (2017) Multi-matrix environmental monitoring to assess heavy element distribution around a municipal solid waste landfill in Italy. *International Journal of Environmental Science and Technology* 14(12), 2591-2602.
- Nimis PL (2016) *The Lichens of Italy. A Second Annotated Catalogue*. EUT, Trieste, 739 pp.
- Nimis PL, Martellos S (2017) ITALIC - The Information System on Italian Lichens. Version 5.0. University of Trieste, Dept. of Biology (<http://dryades.units.it/italic>).
- Nimis PL, Andreussi S, Pittao E (2001) The performance of two lichen species as bioaccumulators of trace metals. *Science of the Total Environment* 275, 43-51.
- Orange A, James PW, White FJ (2001) *Microchemical methods for the identification of lichens*. Twayne Publishers.
- Quevauviller P, Herzig R, Muntau H (1996) Certified reference material of lichen (CRM 482) for the quality control of trace element biomonitoring. *Science of the Total Environment* 187, 143-152.
- Real C, Aboal JR, Fernández JA, Carballeira A (2003) The use of native mosses to monitor fluorine levels - and associated temporal variations - in the vicinity of an aluminium smelter. *Atmospheric Environment* 37, 3091-3102.
- Roos-Barraclough F, Givélet N, Martinez-Cortizas A, Goodsite ME, Biester H, Shotyk W (2002) An analytical protocol for the determination of total mercury concentrations in solid peat samples. *Science of the Total Environment* 292, 129-139.

-
- Shampine WJ (1993) Quality assurance and quality control in monitoring programs. *Environmental Monitoring and Assessment* 26, 143-151.
- Smith CW, Aptroot A, Coppins BJ, Fletcher A, Gilbert OL, James PW, Wolseley PA (2009) *The lichens of Great Britain and Ireland*. London: The British Lichen Society, 1046 pp.
- Stone SF, Freitas MC, Parr RM, Zeisler R (1995) Elemental characterization of a candidate lichen research material—IAEA-336. *Fresenius' Journal of Analytical Chemistry* 352(1), 227–231.
- SW-846 EPA Method 3052, Microwave assisted acid digestion of siliceous and organically based matrices. In: *Test Methods for Evaluating Solid Waste, 3rd Edition, 3rd Update*, US Environmental Protection Agency, Washington DC, 1995.
- Tao S (1995) Kriging and mapping of copper, lead, and mercury contents in surface soil in Shenzhen area. *Water, Air, and Soil Pollution* 83, 161-172.
- Tretiach M, Carpanelli A (1992) Morphology and chlorophyll content as factors influencing the photosynthetic rates of *Parmelia caperata* (L.) Ach. *Lichenologist* 24, 81-90.
- Tretiach M, Candotto Carniel F, Loppi S, Carniel A, Bortolussi A, Mazzilis D, Del Bianco C (2011) Lichen transplants as a suitable tool to identify mercury pollution from waste incinerators: a case study from NE Italy. *Environmental Monitoring and Assessment* 175, 589–600.
- US EPA (2002) *Guidance for Quality Assurance Project Plans*. EPA QA/G-5, Environmental Protection Agency, Washington, DC, USA.
- Wolterbeek HT, Bode P (1995) Strategies in sampling and sample handling in the context of large-scale plant biomonitoring surveys of trace element air pollution. *Science of the Total Environment* 176, 33-43.
- Yafa C, Farmer JG (2006) A comparative study of total and acid-extractable digestion methods for the determination of inorganic elements in peat material by inductively coupled plasma-optical emission spectrometry. *Analytica Chimica Acta* 557, 296-303.

