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Biomonitoring of environmental pollutants with lichens: Data interpretation, methodological aspects and applications

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Not only in research, but also in the everyday world of politics and economics, we would all be better off if more people realised that simple nonlinear systems do not necessarily possess simple dynamical properties.

Robert McCredie May (1976) *Nature* 261

To the holistic maintainers around the world

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ABSTRACT

This research focuses on the methodological standardization of bioaccumulation techniques by lichens. In a first part of this work some major aspects related to the interpretation of biomonitoring results are faced, whereas in the second part, further methodological issues are investigated in a framework of practical applications. The leitmotif of this work is a widely used biomonitor of airborne trace elements and other contaminants, the macrolichen *Pseudevernia furfuracea* (L.) Zopf, that was here repeatedly selected as test species.

Firstly, the background element concentrations for such species were assessed, because their availability is an essential pre-requisite for the correct assessment of trace element pollution. So far, reference values were generally provided for pools of lichen species sharing some functional features (irrespective the proved interspecific bioaccumulation differences), and generally obtained by merging methodologically heterogeneous data from the literature. Differently, here, *P. furfuracea* background values are obtained thanks to a large sampling effort over the Italian territory, hence contemporarily guaranteeing high sample size and methodological uniformity. In a very first contribution, the occurrence of differences in the background elemental composition of the two taxonomical varieties of the target species (*P. furfuracea* var. *furfuracea* and var. *ceratea*) was assessed using samples collected at remote areas. The effect of the variety and that of large-scale environmental factors on the overall element content were explored by multivariate analysis and tested by generalized linear mixed modeling. Intervarietal differences were negligible, confirming that the elemental composition of *P. furfuracea* is mostly affected by local lithology and climatic conditions. After having proved the absence of significant element content differences ascribable to taxonomic traits, in a second contribution, a Principal Component Regression was used to assess whether environmental descriptors were predictive for the lichen elemental content; background levels were finally provided for relatively homogeneous Italian macro-regions. Always in this phase, the analytical outcomes of two different acid mixtures for sample mineralization (i.e., a total digestion with hydrofluoric acid and a partial, *aqua regia*-based one) were compared. The performance of digestions was evaluated by comparing analytical results of experimental samples with the accuracy obtained on aliquots of *P. furfuracea* standard reference material. The total digestion showed a better performance for the determination of Al, As, Ba, Ca, Cd, Cu, Fe, Mn, Ni, Se, Sn and Zn, but not for Cr, Co, P and S. Therefore, two sets of digestion-specific background values were provided, to be alternatively used as methodological homogeneous references in biomonitoring applications, depending on the selected mineralization procedure.

Besides background levels, other approaches can be used to interpret bioaccumulation data. Case in point are the so-called “interpretative scales”. By implementing basic ideas underlying previously available scales, new dimensionless, species-independent scales were developed. Primarily, methodologically consistent element concentration datasets were populated with data from the most recent literature relying on the use of native or transplanted lichens (i.e., passive and active biomonitoring). The scale for native lichens was built up by analyzing the distribution of ratios between element concentration data and species-specific background concentration references (*B* ratio), contextually provided for two foliose lichens (*Flavoparmelia caperata* (L.) Hale and *Xanthoria parietina* (L.) Th. Fr.). Similarly, the scale for transplants was built up by analyzing the

distribution of ratios between element concentration in exposed and unexposed samples (*EU* ratio) of two fruticose lichens (*Evernia prunastri* (L.) Ach. and the well-known *Pseudevernia furfuracea*). The new scales, terminologically revised, overcome most critical points affecting previous ones.

The second part of this research focuses on "smaller-scale" issues with direct implications on the interpretation of biomonitoring results, by addressing the accumulation capacity of elements and polycyclic aromatic hydrocarbons (PAH) in relation to other pollutants and/or to the physiological status of the test species.

In the first contribution, *P. furfuracea* samples were exposed for 6 weeks at sites characterized by different levels of particulate and polycyclic aromatic hydrocarbon (PAH) emissions. Afterwards, samples were transferred to fumigation chambers for 2 weeks, where they were O_3-treated. At each experimental step, three physiological markers (maximum quantum yield of photosystem II, malondialdehyde content and potassium leakage), elemental and PAH concentrations were measured in matched replicate sets. Data were explored by multivariate techniques and the effects of field exposure and ozone (O_3) fumigation were tested by generalized linear models. Besides proving the O_3-tolerance of the test species, it was noticed that the content of some PAHs in highly field-enriched samples, significantly lowered after the ozonation, suggesting a possible role of O_3 in degrading PAHs accumulated by the thallus system, hence opening a scenario of potential interpretative repercussions in transplant-based surveys aimed at evaluating PAH pollution in case of high O_3 ground levels.

The second contribution addresses an aspect that was frequently investigated for mosses, but not for lichens. Indeed, devitalization procedures are standardly carried out on moss monitoring samples, since these enhance the efficiency of elemental capture by passive uptake processes. Such an aspect was never addressed for lichen biomonitors using a large-scale field approach. In this study, the accumulation performance of living and dead *P. furfuracea* samples is assessed through a high sample-sized transplant, by exposing paired sets of living-dead thalli for 8 weeks at 40 sites in a mixed land use area of NE Italy. The two sample sets, consistently described generally low deposition levels over the study area, however, significantly higher accumulation signals were revealed in dead lichens for the majority of tested elements (i.e., Al, Ca, Fe, Ti, As, Cd, Co, Cu, Hg, Pb, Sr, Zn). Moreover, when the sites were classified according to the new bioaccumulation scale for lichen transplants, some interpretational discrepancies arose. In this light, the possibility of sample devitalization should be seriously considered, in order to further contribute to the methodological standardization and harmonization of biomonitoring techniques.

RIASSUNTO

Questa ricerca verte sulla standardizzazione metodologica delle tecniche di bioaccumulo tramite licheni. In una prima parte di questo lavoro vengono affrontati alcuni importanti aspetti relativi all'interpretazione dei risultati del biomonitoraggio, mentre nella seconda parte vengono esaminate ulteriori questioni metodologiche in un quadro di applicazioni pratiche. Il filo conduttore del lavoro è un biomonitor ampiamente utilizzato per la determinazione dei pattern deposizionali di elementi in traccia e altri contaminanti, il macrolichene *Pseudevernia furfuracea* (L.) Zopf, ripetutamente selezionato come specie test.

In primis, vengono determinate le concentrazioni elementari di background per questa specie, dal momento che la disponibilità di tali valori è un prerequisito essenziale per la corretta valutazione dell'inquinamento da elementi in traccia. Finora, tali valori di riferimento erano generalmente forniti per gruppi di specie caratterizzate dalla condivisione di qualche caratteristica funzionale (indipendentemente dalle comprovate differenze di bioaccumulo interspecifiche) e accorpando dati di letteratura metodologicamente eterogenei. In questa sede invece, i valori di background per *P. furfuracea* sono ottenuti grazie ad un intenso sforzo di campionamento sul territorio italiano, garantendo contemporaneamente un'elevata densità campionaria e uniformità metodologica. In un primo contributo vengono testate potenziali differenze a carico della composizione elementare di background delle due varietà tassonomiche delle specie target (*P. furfuracea* var. *furfuracea* e var. *ceratea*) utilizzando campioni raccolti in aree remote. L'effetto della varietà e quello di fattori ambientali a larga scala sul contenuto elementare vengono esplorati tramite tecniche di analisi multivariata e testati con modelli misti lineari generalizzati. Le differenze inter-varietali risultano trascurabili, confermando che la composizione elementare di *P. furfuracea* è principalmente influenzata dalla litologia locale e dalle condizioni climatiche. Dopo aver dimostrato l'assenza di differenze di contenuto elementare significative ascrivibili a tratti tassonomici, in un secondo contributo si utilizza una regressione delle componenti principali per valutare la predittività dei descrittori ambientali sul contenuto elementare del lichene; i livelli di background sono infine forniti per macroregioni relativamente omogenee del territorio italiano. Sempre in questa fase, vengono confrontati i risultati analitici di due diverse miscele acide per la mineralizzazione dei campioni (una digestione totale con acido fluoridrico e una parziale a base di *acqua regia*). Le performance delle digestioni sono valutate confrontando i risultati analitici ottenuti sui campioni sperimentali con l'accuratezza ottenuta su aliquote di materiale certificato di *P. furfuracea*. La digestione totale mostra prestazioni migliori per la determinazione di Al, As, Ba, Ca, Cd, Cu, Fe, Mn, Ni, Se, Sn e Zn, ma non per Cr, Co, P e S. Pertanto, vengono forniti due set di valori di background digestione-specifici, da utilizzare alternativamente come riferimento metodologicamente omogeneo nelle applicazioni di biomonitoraggio, in funzione della procedura di mineralizzazione adottata.

Oltre ai livelli di background, è possibile utilizzare altri approcci per interpretare i dati di bioaccumulo; uno strumento d'ampio utilizzo è costituito per esempio dalle cosiddette "scale interpretative". Implementando le idee alla base delle scale precedentemente disponibili, si sviluppano nuove scale adimensionali e indipendenti dalle specie lichenica. In primo luogo vengono costruiti dataset di concentrazione elementare metodologicamente coerenti utilizzando i più recenti

dati di letteratura ottenuti da licheni nativi o trapiantati (biomonitoraggio passivo o attivo). La scala per i licheni nativi viene costruita analizzando la distribuzione dei rapporti tra i dati di concentrazione elementare e i valori di background specie-specifici (*B* ratio), contestualmente forniti per due licheni fogliosi (*Flavoparmelia caperata* (L.) Hale e *Xanthoria parietina* (L.) Th. Fr.). In modo del tutto simile, la scala per i trapianti viene costruita analizzando la distribuzione dei rapporti tra le concentrazioni elementari in campioni esposti e non esposti (*EU* ratio) di due licheni fruticosi (*Evernia prunastri* (L.) Ach. e la ben nota *Pseudevernia furfuracea*). Le nuove scale, terminologicamente rivisitate, superano i punti più critici che caratterizzavano le precedenti.

La seconda parte di questa ricerca si concentra su ulteriori aspetti con implicazioni dirette sull'interpretazione dei risultati del biomonitoraggio, affrontando lo studio della capacità di accumulo di elementi, ma anche di idrocarburi policiclici aromatici (IPA), in relazione ad altri inquinati atmosferici e/o allo status fisiologico della specie test.

Nel primo contributo, campioni di *P. furfuracea* vengono esposti per 6 settimane in siti caratterizzati da differenti livelli di particolato ed emissioni di IPA e successivamente trasferiti per 2 settimane in camere di fumigazione, dove sono sottoposti a ozonazione. Ad ogni fase sperimentale, e in diversi set di repliche, si misurano tre marker fisiologici (la massima resa quantica del fotosistema II, il contenuto di malondialdeide e la perdita di potassio), il contenuto di elementi e IPA. I dati vengono esplorati tramite tecniche multivariate e gli effetti dell'esposizione in campo e della fumigazione con ozono (O_3) sono testati tramite modelli lineari generalizzati. Oltre a dimostrare l'ozono-tolleranza della specie in esame, si nota che il contenuto di alcuni IPA nei campioni maggiormente arricchiti risulta significativamente ridotto dopo l'ozonazione, suggerendo un possibile ruolo dell'O_3 nel degradare gli IPA accumulati dal sistema biologico e aprendo uno scenario di potenziali ripercussioni interpretative nelle indagini volte alla valutazione dell'inquinamento da IPA in caso di elevate concentrazioni ambientali di O_3.

Il secondo contributo affronta un aspetto frequentemente studiato per i muschi, ma non per i licheni. In effetti, i campioni di muschi destinati al monitoraggio sono normalmente soggetti a procedure di devitalizzazione, poiché queste migliorano l'efficienza di cattura degli elementi mediante processi di captazione passiva. Tale aspetto non è mai stato affrontato per i biomonitor lichenici tramite un approccio di campo su larga scala. In questo studio, le prestazioni di accumulo di campioni vivi e morti di *P. furfuracea* vengono valutate attraverso un lavoro di trapianto caratterizzato da elevate dimensioni campionarie. Set di campioni accoppiati costituiti da talli vivi e morti vengono esposti per 8 settimane in 40 siti in un'area eterogenea dal punto di vista dell'uso del suolo nell'Italia nord-orientale. I due set di campioni descrivono coerentemente i livelli generalmente bassi delle deposizioni nell'area di studio, tuttavia, si rivelano segnali di accumulo significativamente più elevati nei licheni morti per la maggior parte degli elementi testati (i.e, Al, Ca, Fe, Ti, As, Cd, Co, Cu, Hg, Pb, Sr, Zn). Inoltre, quando i siti vengono classificati in base alla nuova scala di bioaccumulo per i trapianti lichenici, emergono alcune discrepanze interpretative. Alla luce di ciò, la possibilità di una devitalizzazione dei campioni dovrebbe essere seriamente presa in considerazione, nell'ottica di un ulteriore contributo alla standardizzazione metodologica e all'armonizzazione delle tecniche di biomonitoraggio.

INTRODUCTION

The lichen system

Lichens are slow-growing symbiotic organisms, traditionally acknowledged as composed of a fungal partner, the mycobiont, and one or more populations of photosynthetic partners, the photobionts (Honegger 2009). The mycobiont constitutes the majority of the biomass in the lichen body (c. 90%), i.e., the *thallus* (Dimijian et al. 2000), and it is generally represented by an ascomycete (Nash 2008). However, the simplistic view of a dual symbiosis has been recently revised, revealing lichens as "open houses" for many microorganisms, such as bacteria, additional algae and fungi (Muggia and Grube 2018, and references therein).
The shape of the lichen thallus is determined by the mycobiont, although it is strongly influenced by the photobiont (Nash 2008). Therefore, lichens often appear as discrete entities. As such, they are treated as individuals in many studies, although they can easily be looked as small ecosystems, exhibiting a still astonishing metabolic, morphological and functional complexity (Tretiach et al. 2013).

Most of lichens are desiccation tolerant (Kranner et al. 2008), being able to survive and recover metabolic activities even if their relative water content decreases below 10% (Farrant et al. 2012). As a matter of fact, lichens lack typical plant structures allowing the regulation of their water content (i.e., stomata and cuticles). As poikilohydric organisms, the water content of lichens completely depends on the atmospheric supply in the form of rainfall, air humidity, fog, and dew (Gauslaa 2014). Similarly, lichens entirely depend on aerial sources to obtain nutrients, that are absorbed over much of their outer surface. As a consequence, many species accumulate from the atmosphere high levels of pollutants (i.e., bioaccumulation; Bajpai et al. 2018; Nimis et al. 2002).
Lichens also produce a wide array of energetically-expensive secondary metabolites, the so-called *lichen substances*. The biochemical properties and the role of a plethora of such compounds are still object of investigation (Huneck 1999). Undoubtedly, lichen substances have antimicrobial, allelopathic, and antiherbivore activity (Lawrey 1986), but they also play a role in metal homeostasis and pollution tolerance (Bhattacharyya et al. 2016).

These perennial organisms, which do not exhibit any seasonal variation, are ecologically important. Indeed, they occur in most terrestrial ecosystems, often as minor contributors (Dimijian et al. 2000). Traditionally presented as pioneer organisms, lichens can make up most of the ground layer biomass in some forests, drylands and tundras, feeling at ease in alpine, polar, and desert habitats (Honegger 2009), but also in managed environments, on heterogeneous types of substrata (trees, rocks, soil, bryophytes and man-made materials; Nimis et al. 2001). Dominating approximately over 8% of the Earth's land surface, lichens have an important - although often overlooked - role as determinants of ecological processes (Aslpund and Wardle 2016).

Lichens as biomonitors of environmental pollution

The impressive suitability of lichens to assess environmental changes *s. lato* is mostly due to their ubiquity over terrestrial ecosystems, their wide range of responses to pollution (lichens vary from extremely poleosensible to poleotolerant), the fact of being perennial, and their ability in absorbing

and accumulating pollutants (Nimis and Purvis 2002). For these reasons, changes in environmental gradients such as atmospheric temperature, humidity, UV radiations, airborne contaminants and pollutants directly affect lichens by inducing responses in terms of biodiversity patterns, species distributions, biomass, physiology, morphology, and bioaccumulation (Cislaghi and Nimis 1997; Gauslaa 2014). Indeed, lichens have been used to track major drivers of atmospheric changes for more than 200 years, with the first observations dating back to the beginning of the industrial revolution (Matos et al. 2017). An exponential spread of lichen biomonitoring studies occurred in the 1960s, when the growing level of sulfur dioxide due to fossil fuel combustion was identified as a major factor influencing lichen growth and distribution (Nimis and Purvis 2002).

Different methodologies can be applied to monitor environmental pollution with lichens, depending on the environmental variables and the spatial scales involved (Nimis 2002).
Traditional bioindication studies focus on the assessment of taxonomic diversity patterns, hence relying on thorough floristic research (Hawksworth 2002). Recently, the rise of functional trait ecology (Laureto et al. 2015) has contributed to shift the focus from the patterns of lichen diversity (e.g., species richness) to those of functional diversity (e.g., Pinho et al. 2011; Matos et al. 2017).
Finally, in bioaccumulation studies, the severity of pollution is assessed in terms of the concentrations of target substances in the lichen matrix (van Dobben 2001). In this case the response evaluation is strictly species-specific, relying on the use of widespread and abundant taxa (Bargagli and Nimis 2002).

Bioaccumulation techniques proved to be very useful in the assessment of depositional patterns of pollutants such as trace elements (Herzig et al. 1989; Bari et al. 2001; Godinho et al. 2008) and polycyclic aromatic hydrocarbons (Augusto et al. 2013; Capozzi et al. 2020). In particular, the use of lichens to assess atmospheric levels, spatial and temporal patterns of trace elements is well-established (Bargagli and Nimis 2002). The positive correlations repeatedly revealed between trace element content in lichens and atmospheric concentrations suggested that such symbiotic organisms are effective in reflecting bulk (wet and dry) depositions (e.g., Pilegaard 1979; Sloof 1995; Bari et al. 2001; Godihno et al. 2008; Loppi and Paoli 2015), especially metal-rich particulate ones (Orsi and Glenn 1991; Bari et al. 2001; Adamo et al. 2007).
Lichen bioaccumulation techniques rely on the use of epiphytic lichens, either native (i.e., autochthonous) or transplanted (involving the collection of bulk material in a relatively unpolluted area, and the subsequent exposure of samples in a target study area for a defined time span), and respectively matching the definitions of “active” and “passive” biomonitoring (Herzig et al. 1989). Recently, the use of lichen transplants has been preferred over the use of native lichens (Brunialti and Frati 2014). As a matter of fact, although native lichens may turn very useful to evaluate deposition levels over long periods (e.g., of the order of one year or more), the transplant technique allows fast and flexible set up of the exposure design, which can be properly realized without the constraints of finding mono-specific autochthonous samples and suitable lichen-carrying trees in heavily polluted areas, where lichens can be rare or even absent (Mikhailova 2002). This ease of application becomes of primary importance with a view to the maximization of the achievable accuracy under the condition of fixed costs (Elzinga et al. 2001). Besides, the knowledge of the lichen exposure time span frees the operators from estimating seasonal / annual growth rates, whose proper assessment is hard-working, being species-specific and severely context-dependent (Fortuna

and Tretiach 2018). On the other hand, transplanted lichens may be affected by the non-native environmental conditions at the transplant sites, which may determine the variation of biological processes related to pollutant accumulation (Tretiach et al. 2011).

Biomonitoring techniques: issues and advantages

The output of biomonitoring studies is different from that obtained through traditional diffusion modelling or active and passive physico-chemical devices. Indeed, while the latter refer to emission data or ambient air concentrations of pollutants, biomonitoring evaluates the biological effects of the pollutants (Markert et al. 2003). Conventional monitoring by instrumental devices obviously does not allow the evaluation of the biological impact of pollution; moreover, monitoring networks by recording instruments are rarely characterized by high densities of measuring points (Bargagli and Mikhailova 2002). By contrast biomonitoring allows a capillary and reliable coverage of the territory (including remote areas) with relatively low costs and without requiring energy supply (Lorenzini 1995). Nonetheless, the operators of biological monitoring have historically faced several criticisms and barriers, which include: (*i*) a poor understanding of the validity of techniques by decisionmakers (possibly related to the poor "commercial inclination" of the biomonitor advocates); (*ii*) the (false) perception that biomonitoring takes longer than chemical monitoring; (*iii*) the belief that biomonitoring is dependent on a particular individual operator (which calls the questionable claim that chemical monitoring could be done by any chemist); (*iv*) the belief that the interpretation of biological data is complex and "uncertain" compared to that of chemical data (Lorenzini 1995). With respect to the latter point, the response of living items is obviously affected by intrinsic biological variability and may reflect several factors other than pollution (Tretiach et al. 2012). In this respect, recognizing the effects caused by "third variables", or separate the effects of intercorrelated variables, may not be an easy task (Nimis and Purvis 2002). However, as far as bioaccumulation techniques are concerned, the achievable high sampling densities, coupled with some methodological precautions, can effectively compensate such variability and reduce the associated uncertainty (Bargagli and Mikhailova 2002).

Despite such protracted distrust, in the latter years biomonitoring techniques are experiencing a growing attention, so much that these have started to be regarded as complementary to conventional instrumental monitoring, as testified by the Directive 2008/50/EC ("Ambient Air Quality and Cleaner Air for Europe"), specifically referring 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).

The need for methodological standardization

Due to the growing importance of quantitative and integrated biomonitoring in risk assessment and environmental decision making (Lionetto et al. 2019), it is more than ever required to obtain highly reliable results potentially expendables in the context of environmental litigation. This can be achieved by producing methodologically robust and homogeneous data, irrespective the contexts of application or, in other words, by implementing processes of methodological standardization over and beyond the national hierarchic level, in order to produce "standard methods"-type documentation (Lorenzini 1995). Indeed, having cross-comparable data produced by different

monitoring surveys is certainly an added value for both research purposes and policy making. Moreover, standardization processes should rely on research efforts aimed at solving baseline methodological issues.

Lichen biomonitoring techniques have sometimes faced such need, especially concerning bioindication approaches (Asta et al. 2002), for which a shared European standard for the assessment of lichen diversity was recently developed (EN 16413:2014). Such a standard is a powerful harmonizing tool describing the actions to be performed in the field and in the laboratory, as well as the procedures to adopt for quality control (EN 16413:2014), although it does not encompass the essential step of result interpretation.

As far as bioaccumulation techniques are concerned, many efforts have been made to solve methodological aspects, ranging from the investigation of (*i*) inter-specific differences in element accumulation capacity (native lichens and transplants: e.g., Nimis et al. 2001; Tretiach and Baruffo 2001; Bergamaschi et al. 2007), (*ii*) the effect of field exposure on element accumulation, as related to meteo-climate, exposure duration and modality in transplants (e.g., Bari et al. 2001; Ayrault et al. 2007), (*iii*) the influence of ecological site-specific factors on lichen growth (commonly applied protocols for native lichens require analyses of parts of thalli c. one-year old, under the postulation that samples of the same size are of the same age; Fortuna and Tretiach 2018); (*iv*) the effect of lichen vitality in relation to the accumulation performance (lichen transplants; Adamo et al. 2007; Capozzi et al. 2020) and that of the sample exposure on lichen vitality (Tretiach et al. 2007).

Despite the great amount of literature targeting methodological aspects for both the techniques of native and transplanted lichens, there is a bewildering array of practices and procedures (from sampling strategy, lichen processing, chemical analyses, quality control and data interpretation), reflecting different "research traditions", local availability of suitable lichen taxa, and an overall lack of national and supra-national standard procedures. Such a lack of consistency often hampers any possibility of comparing different studies and greatly affects data quality.

There are several major methodological issues in bioaccumulation techniques that need to be addressed in order to reliably relate the levels of accumulated pollutants to the magnitude of pollution phenomena, and hence to an environmental "alteration level" (Nimis and Bargagli 1999). For instance, the comparison of experimental values of target pollutants measured in a target lichen matrix with reference ones (i.e., unaltered benchmarks in a "comparable" matrix; Bargagli 1998) is an essential need. Therefore, the availability of species-specific, and up-to-date reference values is a matter of primary importance. Furthermore, bioaccumulation results need to be properly explicated, that calls the need of finely tuned interpretative tools (Nimis and Bargagli 1999). These important aspects can be faced through a "wide-ranging" approach, encompassing field work and meta-analysis of available data, to comparable degrees. Moreover, further essential methodological points strictly deal with the functioning of the lichen symbiosis. These "smaller-scale" issues can be addressed in a fully experimental-based frame. Cases in point are the assessment of the response of biomonitors to mixtures of pollutants, and the understanding of interaction phenomena possibly occurring between accumulated target and non-target substances at the thallus system level (Kodnik et al. 2015). The effects of inherent physiological states of biomonitors on the measured concentrations of target compounds also deserve to be further investigated, because the lichen health status may act as an additional confounder, possibly altering accumulative dynamics and

performances. Indeed, such an aspect was deeply addressed in the context of biomonitoring by mosses (e.g., Giordano et al. 2009; Deben et al. 2016), but it was only scarcely investigated for lichens, irrespective the applied experimental approach (Nieboer et al. 1976; Chettri et al. 1997; Adamo et al. 2007).

It is worth to notice that the lack of methodologically homogenous reference values and interpretative tools, along with the missing acknowledgement of lichen inherent processes affecting accumulation behaviour, may determine the failure of the basic assumption of bioaccumulation, that is that pollutant concentrations measured in lichen matrices are able to *finely* reflect the atmospheric ones. In this light, the studies presented in this thesis, organized in two distinct modules (Part 1 and Part 2), face such still open or not fully enucleated issues.

The first part of this work is entitled "*Interpretational Standardization in Bioaccumulation Techniques*" and includes four studies: general aims were to provide reliable background reference values for trace elements in a widely used lichen biomonitor and to construct new interpretative tools for bioaccumulation data based on the most recent available data.

The first three studies can be regarded as contributions to the setting up of a novel approach for the proper derivation of background element content values (BECs) in lichens. Indeed, these works cover different aspects related to the BEC assessment of an illustrious biomonitor, the macrolichen *Pseudevernia furfuracea* (L.) Zopf. The choice of this species was driven by its peculiar morphological, ecological and metabolic features. Indeed, its great abundance (Martellos 2003), the high surface per mass unit (Tretiach et al. 2005), and its good response to transplantation (Tretiach et al. 2011) have made *P. furfuracea* an extensively exploited bioaccumulator in native (e.g., Garty and Ammann 1987; Stratis et al. 2009; Nascimbene et al. 2014) and transplant applications (e.g., Jozic et al. 2009; Tretiach et al. 2011; Petrova et al. 2015; Kodnik et al. 2015, 2018).

The first study, "*Infraspecific variability in baseline element composition of the epiphytic lichen Pseudevernia furfuracea in remote areas: Implications for biomonitoring of air pollution*", is a deepening investigation of an acknowledged aspect of bioaccumulation in lichens, that is that different taxa may exhibit different bioaccumulation capacity, and possibly, different baseline element content in "pristine" areas. Usually, differences in elemental composition were acknowledged at the interspecific level (e.g., Nimis et al. 2001; Bergamaschi et al. 2007), but these were also recently hypothesized for *P. furfuracea* at the infraspecific level (Malaspina et al. 2014). As a matter of fact, this species has two morphologically identical varieties, differing for the lichen substance profile and their distribution at both supra-regional (Hale 1956; Hawksworth and Chapman 1971) and regional scale (e.g., in the Alps the varieties exhibit an interesting altitudinal vicariance; Martellos 2003). In this first contribution, the hypothesis of a different varietal element content composition in remote areas is tested using paired samples of the two varieties collected at background sites in the main Italian mountain ranges, in order to solve the issue of their joint use in bioaccumulation applications.

In the second work, "*Background element content of the lichen Pseudevernia furfuracea: A supra-national state of art implemented by novel field data from Italy*", the methodological variability in lichen bioaccumulation surveys carried out with *P. furfuracea* in Europe is thoroughly screened, a process also determining the construction of a publicly available repository of literature bioaccumulation data (http://dsv.units.it/it/ricerca/prodotti-ricerca/Software-e-banche-dati). Finally,

BECs for this species are assessed by considering original field data from a large sample collection carried out over the entire Italian territory.

In the third study, "*Background element content in the lichen Pseudevernia furfuracea: A comparative analysis of digestion methods*", coupled *P. furfuracea* samples from remote areas are subjected to different acid extraction protocols (i.e., sample mineralization) in order to quantify the effects of this analytical step on BEC levels. Indeed, the acid extraction, as affecting the accuracy of the analytical procedures for several elements, may alter the results of elemental determination in biological matrices (Rodushkin et al. 1999; Baffi et al. 2002; Ashoka et al. 2009; Rashid et al. 2016). Nonetheless, details on acid digestion are often missing in lichen biomonitoring literature, thus possibly introducing a bias in reference values assessed considering data from highly heterogeneous sample digests (e.g., Bargagli 1998). For this reason, besides testing the effect of sample mineralization, two distinct, digestion-specific sets of BEC values are provided for *P. furfuracea* in Italy.

The last study included in the first part of this thesis deals with the construction of new, methodological uniform tools for the interpretation of bioaccumulation data. The work entitled "*New interpretative scales for lichen bioaccumulation data: The Italian proposal*" is the product of the efforts of the Working Group on Biomonitoring of the Italian Lichen Society, that has recently drafted the "Italian Guidelines for the Use of Lichens as Bioaccumulators" (ready to be published online by the Italian Institute for Environmental Protection and Research, ISPRA), which include two standard methods relying on native and transplanted lichens. In this contribution, the crucial point of the construction of interpretative tools for bioaccumulation data is faced, given the important outcomes for decision making and environmental forensics (Hays et al. 2007; Clewell et al. 2008).

Previously available interpretative tools consisted in (*i*) ordinal scales referring levels of naturality / alteration to specific ranges of elemental concentrations, as derived from the analysis of literature data (native lichens; Nimis and Bargagli 1999), and (*ii*) a fully expert-assessed scale based on dimensionless ratios between post- and pre-exposure element concentration values (transplants; Frati et al. 2005). Moving from the core ideas of these scales, in this study, a new collection of methodological uniform lichen element content data lead to the implementation of two revised "bioaccumulation scales" for native and transplanted lichens.

The second part of this thesis is entitled "*Methodological issues in practical applications*". Two studies are presented under this banner, reporting the results of transplant applications always carried out with the macrolichen *P. furfuracea*. Here, two "small-scale" aspects with possible repercussions on the interpretation of biomonitoring results are faced in an experimental frame.

In the study "*Beyond ozone-tolerance: Effects of ozone fumigation on trace element and PAH enriched thalli of the lichen biomonitor Pseudevernia furfuracea*", the effects of a strongly oxidizing secondary pollutant (ozone, O_3), elements and polycyclic aromatic hydrocarbons (PAHs) on the physiology of *P. furfuracea* are investigated after the exposure of samples in the field and their subsequent ozonation in a controlled environment (fumigation chambers). The tolerance of chlorolichens to O_3 was previously observed (e.g., Bertuzzi et al. 2013, 2018; Pellegrini et al. 2014), however, in this case, *P. furfuracea* samples were preliminary subjected to a mixture of multi-origin pollutants (i.e., thalli were enriched in heavy metal-rich particulate, PAHs, etc) to test the

hypothesis that field-stressed thalli could exhibit physiological impairment due to subsequent ozonation.

In this context, an interesting significant decrease of bioaccumulated PAHs after the ozonation is highlighted, and tentatively interpreted as a depletion effect caused by O_3, hence suggesting the possibility of a misinterpretation of PAH levels in case of high ambient air concentrations of O_3.

The last work presented, "*Element accumulation performance of living and dead lichens in a large sample-sized transplant application*", deals with the assessment of the interpretative bias associable to the use of living and dead *P. furfuracea* samples. The fact that dead matrices are able to better accumulate the elemental fraction associated to particulate depositions is not actually new, as intensively investigated for mosses (e.g., Fernández et al. 2009; Deben et al. 2016), but to a substantially lesser extent for lichens (Adamo et al. 2007). In this study the issue is faced by implementing a large sample-sized transplant survey, taking advantage of the availability of the new interpretative scale (see *supra*), that allows to quantify the interpretational bias of bioaccumulation results caused by lichen physiological states, in relation to the specific pollutant loads affecting the study area.

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PART 1

INTERPRETATIONAL STANDARDIZATION IN BIOMONITORING TECHNIQUES

Infraspecific variability in baseline element composition of the epiphytic lichen *Pseudevernia furfuracea* in remote areas: implications for biomonitoring of air pollution

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Abstract

The epiphytic lichen *Pseudevernia furfuracea* is widely used as biomonitor of airborne trace elements and other contaminants, and consists of two taxonomic varieties (var. *furfuracea* and var. *ceratea*).

Here we assessed the occurrence of inter-varietal differences in the elemental composition of paired samples of var. *furfuracea* and var. *ceratea* collected in 20 remote sites of Italian mountains. The concentration of 40 elements was measured by Inductively Coupled Plasma Mass Spectroscopy, after digestion with HNO_3 and *aqua regia*. The magnitude of inter-varietal differences compared to the effect of large-scale site-dependent environmental factors (i.e. lithological substrate, host tree species and altitude) on overall element content was explored by multivariate analysis techniques and tested by Generalized Linear Mixed Modeling (GLMM). Further GLMMs were separately fitted for each element testing taxonomic-related variability against uncertainty associated to the analytical procedure.

Inter-varietal differences were statistically significant only for Hg and P, with higher content in var. *ceratea* at most sites, and for Mg and Zn, showing the opposite pattern. Since the elemental composition of *P. furfuracea* in remote sites was mostly affected by local lithology and climatic conditions, our results confirm that lichen material for active biomonitoring should be collected in a single ecologically homogeneous remote area. We also indicate sites in the Eastern Alps where *P. furfuracea* showed the minimum content of most elements, which are suggested as locations to collect lichen material for transplants.

Besides the context-dependency at large spatial scale, variations of elemental composition apparently related to taxonomy, could possibly be due to unequal incidence of morphological traits of the collected material. Further research is needed to clarify this issue, and how it affects bioaccumulation phenomena.

Keywords: trace elements; atmospheric depositions; baseline values; *ceratea*; *furfuracea*; lichen transplants; taxonomy.

1. Introduction

Lichens have reliably been used as bioaccumulators of air pollutants in pristine, near-natural habitats (Loppi 2014), agrisystems (Will-Wolf et al. 2015), and urban, industrial or mixed areas (Adamo et al. 2003; Bargagli et al. 1997). In the latter cases, the monitoring surveys focused on hotspot emission sources (Tretiach et al. 2011), or extended to regional scale (Sawidis et al. 1995; Nimis et al. 2000), are suitable tools for environmental impact assessment and air quality monitoring programs.

Due to peculiar morphological and physiological traits, the element composition of lichens reflects the chemical composition of the air (Bargagli 1998; Nash 2008) and a relationship between element concentrations in thalli and in bulk atmospheric depositions has repeatedly been reported (e.g. Herzig et al. 1989; van Dobben et al. 2001). However, the bioaccumulation of airborne elements depends on several controlling factors (Garty 2001), ranging from the metabolic state of the thallus (Godinho et al. 2008) and the meteoclimatic conditions at the exposure sites (Ayrault et al. 2007), to the chemical composition in secondary metabolites, the so-called "lichen substances", which might explain observed species-specific differences (Hauck and Huneck 2007). Recently, Malaspina et al. (2014) suggested possible differences in baseline elemental composition and bioaccumulation capacity of the two varieties of *Pseudevernia furfuracea* (L.) Zopf related to differences of the secondary metabolite profile, thus raising the issue that taxonomic-related traits at infraspecific level might affect the bioaccumulation results. In Europe *P. furfuracea* consists of two morphologically indistinguishable varieties, *P. furfuracea* var. *furfuracea* (L.) Zopf (with physodic and oxyphysodic acids) and *P. furfuracea* var. *ceratea* (Ach.) Hawksw. (with physodic and oxyphysodic acids variably present, but with olivetoric acid, absent in the typical variety) (Ferencova et al. 2010). The two varieties can be distinguished by a simple spot test with diluted sodium hypochlorite (C-test, Elix and Stocker-Wörgötter 2008), which gives a positive, red color in presence of olivetoric acid.

This lichen has widely been used in a number of biomonitoring surveys, mostly based on the transplant technique, in which thalli of suitable species are collected from remote sites and exposed in target areas for assessment of depositions of trace elements (e.g. Sloof 1995; Frati et al. 2005) and organic compounds (e.g. Kodnik et al. 2015), on account of its local abundance (Martellos 2003), good resistance to gaseous phytotoxic pollutants and climatic stresses (Marti 1983; Miszalski and Niewiadomska 1993; Tretiach et al. 2007), and its capacity in heavy metal accumulation and sequestration (Adamo et al. 2007; Ates et al. 2007).

Considering the significance of this species in the biomonitoring of atmospheric pollutants and the need of methodological standardization of this technique, the issue of a possible intervarietal difference in elemental composition is particularly relevant, although only very few authors reported the indication of varieties used in the biomonitoring surveys (e.g. Rinino et al. 2005; Tretiach et al. 2007, 2011).

Here we provide for the first time a multi-site, multi-element assessment of the baseline element content in the target species under proximate-natural environmental conditions. Our specific aims are to: (*i*) assess the occurrence of possible differences in the elemental composition of *P. furfuracea* attributable to taxonomically-related traits at varietal level, (*ii*) test for 40 chemical

elements the magnitude of inter-varietal differences against the overall effect of large-scale site-dependent environmental factors including location, lithology, and host tree species.

2. Materials and methods

2.1. Study sites and sampling

Pseudevernia furfuracea is a fruticose, meso-xerophilous and photophilous macrolichen, with a cool-temperate to boreal-montane distribution (Rikkinen 1997; Smith et al. 2009), particularly abundant in the coniferous forests of the Alps, rarer along the Apennines (Nimis and Martellos 2002), occurring mainly on acidic, non-eutrophicated bark. For this study, the lichen material was collected in 20 sites (Supplementary Fig. S1), at middle-high elevation (Table 1). In the field, the appraisal of the co-occurrence of var. *furfuracea* and var. *ceratea* was performed by spot-test on randomly selected thalli. At each site, a minimum of 30 thalli were sampled whenever possible from the same tree species (Table 1), preferring those located on branches under the tree canopy compared to the bole, in order to control for potential confounders of elemental composition related to exposure, light radiation, and atmospheric particulate deposition (Adamo et al. 2008).

Table 1 List of the sampling sites of the two varieties of the epiphytic lichen *Pseudevernia furfuracea*, var. *furfuracea* and var. *ceratea*, with location, lithological substrate and tree species.

ID	Province	Latitude (N)	Longitude (E)	Altitude (m)	Substrate	Tree species
1	Torino	44°54'52.96"	7°08'29.74"	1560	Metamorphic siliceous	*Larix decidua*
2	Torino	44°54'11.92"	7°08'07.26"	1780	"	"
3	Torino	44°53'25.63"	7°07'50.48"	2100	"	"
4	Bergamo	46°02'59.06"	9°46'12.34"	1951	"	*Pinus cembra*
5	Sondrio	46°04'06.25"	9°45'55.52"	2000	"	"
6	Sondrio	46°16'51.18"	9°53'50.19"	1250	"	*Larix decidua, Picea abies*
7	Sondrio	46°18'19.53"	9°56'13.42"	1990	"	*Larix decidua*
8	Sondrio	46°18'07.47"	9°56'11.83"	2120	"	*Larix decidua, Pinus cembra*
9	Trento	46°02'34.73"	11°02'17.82"	1635	Limestone	*Larix decidua*
10	Bolzano	46°50'03.19"	11°18'09.82"	1720	Metamorphic siliceous	*Picea abies*
11	Bolzano	46°49'54.19"	11°19'01.68"	1820	"	"
12	Bolzano	46°50'06.14"	11°18'41.79"	1845	"	"
13	Belluno	46°24'57.93"	11°53'03.55"	1835	Limestone	*Larix decidua, Picea abies*
14	Belluno	46°24'48.78"	11°52'48.83"	1965	"	*Larix decidua, Picea abies*
15	Udine	46°25'47.67"	12°40'55.96"	1650	Sandstone	*Larix decidua*
16	Udine	46°25'35.59"	12°41'08.72"	1850	"	"
17	Udine	46°25'20.88"	12°41'02.01"	1970	"	"
18	Udine	46°28'01.06"	13°41'35.31"	1830	"	"
19	Lucca	44°12'10.29"	10°21'32.17"	1230	Limestone	"
20	Catanzaro	39°19'26.07"	16°28'02.44"	1600	Intrusive siliceous	*Pinus nigra laricio*

In the field, disposable latex gloves were used throughout the sampling operations to avoid thalli contamination. Samples were detached from the substratum, placed into paper envelops and sealed in plastic bags. In the laboratory, the lichen material was dried out at room temperature until reaching constant mass. All thalli were sorted according to the infraspecific variety, which was assessed by repeated C spot tests, taking extreme care in the subdivision of the intermingled thalli.

A further check was performed on problematic samples by means of TLC (Culberson 1972; Elix and Ernst-Russell 1993). All thalli were carefully cleaned from fragments of tree bark, other lichens and mosses, and arthropods using powder free gloves and plastic tweezers. Terminal lobes homogenous in size (15-25 mm), shape, and isidia density (visually assessed) were selected, separated from the thallus using ceramic scissors, and pulverized through a planetary ball mill (Retsch PM100), with milling cycles of 4 minutes at 550 rpm. From 3 to 5 aliquots (i.e. technical replicates) of 1 g each were obtained for both varieties and each site. Overall, 171 technical replicates were obtained, 94 of which belonging to var. *furfuracea*, and 77 to var. *ceratea* (Supplementary Table S1).

2.2. Analytical procedures

All technical replicates were subjected to multielemental analysis at ACME Analytical Laboratories (Vancouver, Canada), purposely selected as widely cited in the literature as high-quality data provider for element content in different matrices, including lichens (e.g. Gür and Yaprak 2011; Tretiach et al. 2011b; d'Abzac et al. 2013; Ordóñez et al. 2013; Keshavarzi et al. 2015; García-Ordiales et al. 2016). Lichen material was submitted to acid digestion by two subsequent treatments: (*i*) ACS-grade HNO_3 for 1 h; (*ii*) aqua regia (ACS-grade HCl:HNO_3 in a volume ratio of 3:1) in a boiling water bath (95 °C) for 1 h. The concentration of 53 elements (Ag, Al, As, Au, B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Fe, Ga, Ge, Hf, Hg, In, K, La, Li, Mg, Mn, Mo, Na, Nb, Ni, P, Pb, Pd, Pt, Rb, Re, S, Sb, Sc, Se, Sn, Sr, Ta, Te, Th, Ti, Tl, U, V, W, Y, Zn, Zr) was determined through ICP-MS with a Perkin Elmer Elan 6000 ICP mass spectrometer. Not all elements are decomposed during the digestion (Fletcher 1981), but for the purposes of the study, the obtained results were considered as total concentrations. The values were expressed on a dry mass basis (μg g^{-1}). ACME QA/QC protocol incorporates a sample-prep blank carried through all stages of preparation and analysis as the first sample, a pulp duplicate to monitor analytical precision, two reagent blanks to measure background and aliquots of in-house Standard Reference Materials like V16 and CDV-1 (plant leaves) to monitor accuracy. Both standards were certified in-house against 38 Certified Reference Materials. Detection limits of the analytical procedure for each element are reported in Supplementary Table S2. Accuracy and precision of the analytical procedures were mostly satisfactory, with 100 % recovery of element content in reference standards being always included in the 95 % confidence interval of analytical results (Supplementary Table S2). The only exceptions were S and Se, whose concentrations were over- and under-estimated by 10 % and 30 %, respectively. The standard reference material CRM 482 'lichen' *P. furfuracea* was also sent to ACME to be blindly analysed. It should be considered that certified and indicative values for such standard were obtained by using a mixture of HNO_3 and HF at 100°C for 14 h and 150°C for 2h (Quevauviller et al. 1996), making the assessment of QC problematic due to the different digestion protocols. However, for comparative purposes, expected and measured element content values, as well as recovery percentages, are also reported for such certified material in Supplementary Table S2.

2.3. Data analysis

Elements showing null variance in either lichen variety across the 20 sites (Be, In, Re, Te), as well as those below the threshold value for analytical detection (B, Ga, Pd, Pt, Ta, Tl, U, V, W), were excluded from the data analysis, which was performed for the remaining 40 elements.

A data matrix of mean element content calculated over the technical replicates of each lichen sample (i.e. material of either variety collected in a given site) was constructed, with data standardized for each element in order to account for scale differences. The matrix was submitted to Cluster Analysis (CA, separately performed for elements and lichen samples using Euclidean distance and Ward's clustering algorithm) and Principal Component Analysis (PCA) to explore elemental composition of lichen samples related to taxonomic variety and environmental conditions at the sampling sites. For interpretation purpose, different site-related factors possibly associated to lichen element content, such as location (latitude, longitude, altitude), lithology and tree species, as well as lichen variety, were included in the PCA as supplementary variables (i.e. plotted in the multivariate space, but not used to calculate the principal components), following the approach suggested by Legendre and Legendre (1998). Categorical supplementary variables were implemented considering each level as a binary "dummy variable", assuming values 1 or 0 according to the level occurrence in the observations.

In order to disentangle the effects of context-dependent environmental factors and taxonomic traits related to lichen variety on elemental composition, two Generalized Linear Mixed Models (GLMM) were fitted. First, main and interactive effects of lichen variety (fixed effect with two levels, either *furfuracea* or *ceratea*), cluster of samples (random effect) and group of elements (random effect) were tested, considering element content as the dependent variable. In particular, standardized values for each element were used, separately considering each element content value as an individual observation.

In this way, differences of geographical distribution among homogenous groups of elements, as well as differences of elemental composition among homogeneous clusters of samples, as resulting from exploratory multivariate analyses, were tested for statistical significance. At a preliminary stage, different GLMMs were considered including different combinations of cluster of samples and groups of elements, obtained by cutting the corresponding dendrograms at different threshold values of Euclidean distance, in order to assess the result consistency in relation to possible artifacts due to the specific combination.

In a second, more detailed analysis, a GLMM was fitted for each chemical element considering raw content data as the dependent variable and main and interactive effects of lichen variety (fixed effect, two levels) and sample (random effect) as independent variables. In this way, within-sample variability assessment was based on technical replicates, specifically relating to uncertainty associated to the digestion and ICP-Mass spectrometric analysis, while preventing from inference about within-site data variability associated to micro-environmental factors.

Pair-wise comparisons from the GLMMs were tested with the LSD post-hoc test. The threshold value of p for statistical significance was set to $\alpha' = \alpha/N$ with N = number of comparisons, following the Bonferroni's correction method. p-values ranging between α' and α were considered marginally significant. All data analyses and graphics were performed with the software package Statistica 7 (StatSoft Inc., Tulsa).

3. Results

3.1. Chemical composition of lichen samples and multivariate analysis

In general, lichen samples showed a rather homogeneous chemical composition. The utmost differences were an enhanced accumulation of some metals such as Hg, Pb, Sb, Sn and Zn in samples from Western Alps and of lithophile elements such as Al, Fe, Li and Ti in those from the Southern Apennines site (Supplementary Table S3).

Three groups of chemical elements were identified by CA (Fig. 1a). CA of the lichen samples (Fig. 1b) showed three main clusters, mostly corresponding to the geographical location and environmental conditions of the collection sites, but not to lichen variety. Cluster I included 18 samples mostly from the Eastern Alps (Fig. 1b, Table 1), on *Larix decidua*, over limestone or sandstone substrates. Cluster II included 20 samples (Fig. 1b, Table 1), mostly from the Western Alps, on different tree species, over metamorphic siliceous rock. Cluster III was limited to the samples of both *P. furfuracea* varieties from Southern Apennines (Fig. 1b, Table 1).

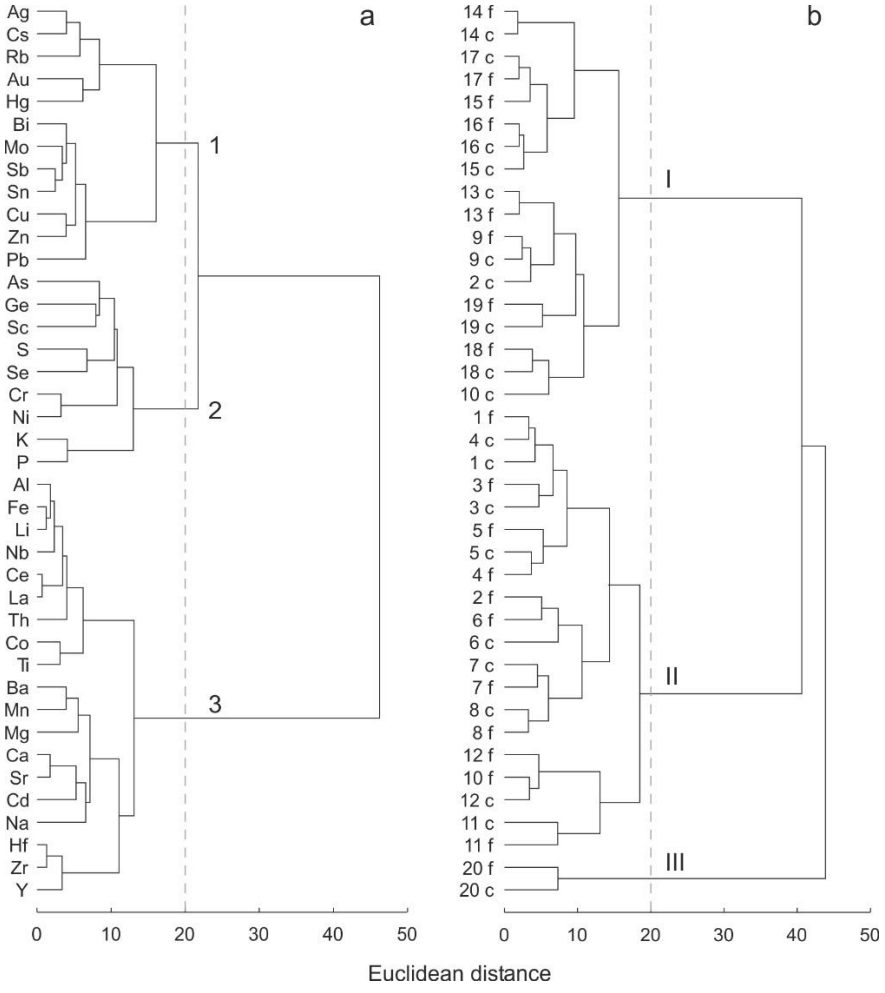

Fig. 1 Dendrograms of elements (a) and lichen samples (b). Sample labels refer to combinations of site number (Table 1) and lichen variety ("f "and "c" for var. *furfuracea* and *ceratea*, respectively). Arabic (1 to 3) and roman (I to III) numerals refer to groups of elements and cluster of sites, respectively.

The PCA results showed that lichen variety was not associated with either the ordination axes (Fig. 2a), or the groups of chemical elements (Fig. 2b). Differently, longitude, sandstone and limestone

substrates, and *L. decidua* were negatively associated to the second ordination axis, and positively with the elements of group 1, while latitude showed positive correlation with the first ordination axis, and negative with the elements of group 3 (Fig. 2b). Intrusive siliceous substrates and *P. nigra* subsp. *laricio*, peculiar to the samples from the single site of Southern Apennine, were negatively associated with the first ordination axis, and positively with the elements of group 3 (Fig. 2b). The plot of lichen samples in the ordination space reflected the element distribution, with samples of clusters I and II positively and negatively associated with the second ordination axis, respectively (Fig. 2c), and the samples of cluster III well separated from all the other samples (Fig. 2c).

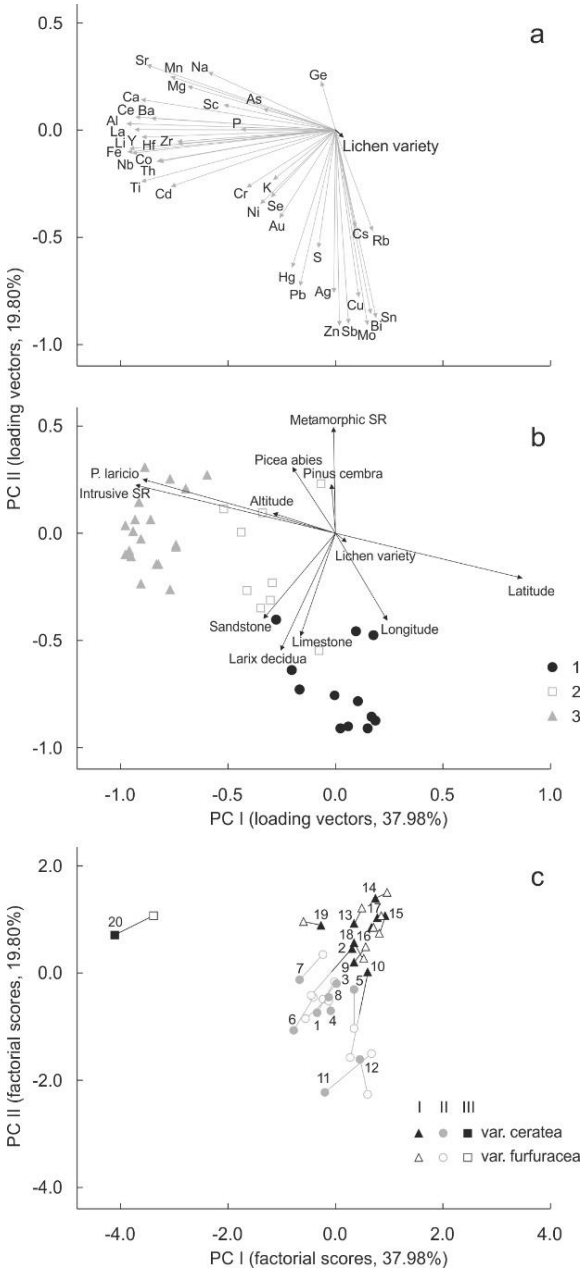

Fig. 2 PCA of 40 chemical elements in samples of *P. furfuracea* at 20 remote sites. (a) Loading vectors of elements. Lichen variety (black arrow) is also plotted as a supplementary variable, following Legendre and Legendre (1998). (b) Elements symbolized according to the three main groups (1, 2, 3) resulting from CA (Fig. 1a); geological substrate, tree species and geographical coordinates of sites are plotted as supplementary variables. (c) Factorial scores of samples, indicated with different symbols according to the three clusters (I, II, III) resulting from CA (Fig. 1b), and to lichen variety. Paired samples of the two varieties at each site (numbers) are connected by solid lines representing Euclidean distances in the PC space.

3.2. Infraspecific and site-dependent effects on elemental content of *P. furfuracea*

Our first GLMM analysis confirmed that the overall element content was not affected by the lichen variety (non-significant first order and interactive effects, Table 2). Cluster of samples essentially corresponding to geographical macroareas did not show significant differences of overall elemental content (p = 0.17, for the first order effect of the cluster of samples, Table 2), but such differences were highly significant when limited to specific groups of elements (p = 0.0011 for the interaction of cluster of samples and group of elements, Table 2).

In particular, the samples of cluster III (i.e. the southernmost site) showed by far the highest content of lithophile elements belonging to group 3 (Fig. 3). Differently, the content of elements of groups 1 and 2 was consistently lower in the Eastern Alps, but higher in the Western Alps (Fig. 3). Significant differences also occurred, within each cluster of samples, between elements of different groups (Fig. 3).

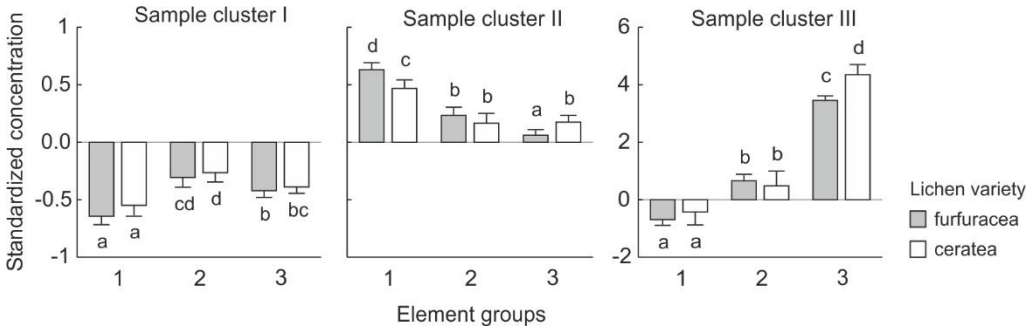

Fig. 3 Context-dependency of *P. furfuracea* elemental composition. Data refer to mean and 95 % confidence interval of standardized element content in clusters of samples, for 3 different groups of elements (see Fig. 1 for details on groups and clusters). Letters above bars indicate significant differences within each cluster of samples (post-hoc LSD test for the interactive effect of element group, sample cluster and variety from the GLMM in Table 2).

Interestingly, the third order interaction, was statistically significant (p = 0.0002, Table 2), showing that, differences of element content between *P. furfuracea* var. *furfuracea* and var. *ceratea*, even though not systematically observed throughout the dataset, still occurred within restricted geographical and environmental contexts, represented by specific combinations of samples and element groups (Fig. 3).

Table 2 Summary of the general linear mixed model (GLMM) testing for main and interactive effects of lichen variety, cluster of samples and group of elements on element concentration in the epiphytic lichen *Pseudevernia furfuracea*. See Figs. 1 and 2 for further details on clusters and groups. Significant effects (p < 0.05) are reported in italic.

Factor	Effect type	SS	d.f.	MS	F	p
Variety (V)	Fixed	3.34	1	3.34	0.81	0.47
Cluster of samples (S)	Random	977	2	489	2.89	0.17
Group of elements (E)	Random	452	2	226	5.42	0.061
V × S	Random	6.39	2	3.19	0.96	0.46
V × E	Random	8.58	2	4.29	3.49	0.067
S × E	Random	707	4	177	51.3	*0.001*
V × S × E	Random	13.8	4	3.45	5.56	2×10^{-4}

Our detailed GLMMs on single element content provided generally consistent results across all the 40 elements (Table 3), being mostly unaffected by lichen variety, with 4 exceptions: P, Hg, Mg, and Zn (Table 3). Paired samples for these elements showed an interesting pattern, with intervarietal differences variable in magnitude and direction (Fig. 4). For most remaining elements, a significant interaction effect of lichen variety and sample (Table 3) indicated the occurrence of inter-varietal differences limited to specific *P. furfuracea* samples and, given the non-significant first order effect of lichen variety, evenly distributed between the two varieties. For example, As and Ni, two elements of high environmental concern, showed low values in both varieties in most samples, with significantly higher values in either variety in a limited number of cases (Fig. 4).

Table 3 Summary of the general linear mixed model (GLMM) testing for main and interactive effects of lichen variety (V) and sample (S) on the concentration of 40 chemical elements in the epiphytic lichen *Pseudevernia furfuracea* (*p*-values < 0.05 are reported in italic).

Effect	**d.f.**	**SS**	**MS**	**F**	***p***	**SS**	**MS**	**F**	***p***	**SS**	**MS**	**F**	***p***	**SS**	**MS**	**F**	***p***
			Ag				**Al**				**As**				**Au**		
V	1	1.3×10^{-8}	1.3×10^{-8}	3×10^{-4}	0.99	32.5	32.5	0.007	0.93	0.003	0.003	0.18	0.68	0.004	0.004	0.16	0.69
S	19	0.014	7×10^{-4}	14.4	*$<10^{-4}$*	2.1×10^{6}	1.1×10^{5}	21.8	*$<10^{-4}$*	1.03	0.054	2.91	*0.012*	1.59	0.084	2.59	*0.022*
V × S	19	9×10^{-4}	5.2×10^{-8}	16.2	*$<10^{-4}$*	9.8×10^{4}	5154	4.25	*$<10^{-4}$*	0.35	0.019	2.35	*0.002*	0.61	0.032	41.3	*$<10^{-4}$*
			Ba				**Bi**				**Ca**				**Cd**		
V	1	1.23	1.23	0.11	0.75	9.0×10^{-6}	9.0×10^{-6}	0.023	0.89	3.9×10^{6}	3.9×10^{6}	1.17	0.29	0.005	0.005	3.55	0.074
S	19	3498	184	13.0	*$<10^{-4}$*	0.058	0.003	5.23	*4×10^{-4}*	1.4×10^{9}	7.4×10^{7}	18.3	*$<10^{-4}$*	0.39	0.021	11.1	*$<10^{-4}$*
V × S	19	269	14.1	52.6	*$<10^{-4}$*	0.011	0.001	3.84	*$<10^{-4}$*	7.6×10^{7}	4.0×10^{6}	59.8	*$<10^{-4}$*	0.035	0.002	17.6	*$<10^{-4}$*
			Ce				**Co**				**Cr**				**Cs**		
V	1	0.006	0.006	0.17	0.68	0.001	0.001	0.46	0.50	0.001	0.001	0.001	1.00	2.4×10^{-5}	2.4×10^{-5}	0.017	0.90
S	19	12.3	0.65	15.0	*$<10^{-4}$*	1.34	0.070	20.7	*$<10^{-4}$*	69.2	3.64	12.3	*$<10^{-4}$*	1.04	0.055	32.1	*$<10^{-4}$*
V × S	19	0.82	0.043	17.2	*$<10^{-4}$*	0.065	0.003	7.82	*$<10^{-4}$*	5.64	0.30	10.0	*$<10^{-4}$*	0.032	0.002	33.5	*$<10^{-4}$*
			Cu				**Fe**				**Ge**				**Hf**		
V	1	40.0	40.0	3.96	0.061	3706	3706	0.37	0.55	2.2×10^{-5}	2.2×10^{-5}	1.06	0.31	0.003	0.003	1.47	0.24
S	19	1058	55.7	4.53	*9×10^{-4}*	2.9×10^{6}	1.5×10^{5}	12.6	*$<10^{-4}$*	0.001	7.3×10^{-5}	3.50	*0.004*	0.10	0.005	2.17	*0.049*
V × S	19	2334	12.3	74.5	*$<10^{-4}$*	2.3×10^{5}	1.2×10^{4}	13.9	*$<10^{-4}$*	4×10^{-4}	2.1×10^{-5}	0.99	0.48	0.048	0.003	19.3	*$<10^{-4}$*
			Hg				**K**				**La**				**Li**		
V	1	0.036	0.036	7.69	*0.012*	3.3×10^{4}	3.3×10^{4}	0.26	0.61	0.001	0.001	0.16	0.69	0.004	0.004	0.96	0.34
S	19	0.42	0.022	3.87	*0.002*	3.5×10^{7}	1.8×10^{6}	12.8	*$<10^{-4}$*	2.34	0.12	12.7	*$<10^{-4}$*	1.37	0.072	15.9	*$<10^{-4}$*
V × S	19	0.11	0.006	46.7	*$<10^{-4}$*	2.8×10^{6}	1.4×10^{5}	5.23	*$<10^{-4}$*	0.18	0.01	21.8	*$<10^{-4}$*	0.086	0.005	5.67	*$<10^{-4}$*
			Mg				**Mn**				**Mo**				**Na**		
V	1	7.6×10^{4}	7.6×10^{4}	6.56	*0.019*	86.4	86.4	0.35	0.56	0.017	0.017	2.92	0.10	94.0	94.0	0.23	0.64
S	19	4.8×10^{6}	2.5×10^{5}	18.1	*$<10^{-4}$*	1.1×10^{5}	5843	19.4	*$<10^{-4}$*	2.27	0.12	16.8	*$<10^{-4}$*	1.2×10^{5}	6556	13.3	*$<10^{-4}$*
V × S	19	2.7×10^{5}	1.4×10^{4}	13.0	*$<10^{-4}$*	5733	302	29.1	*$<10^{-4}$*	0.13	0.007	19.5	*$<10^{-4}$*	9378	493.6	20.3	*$<10^{-4}$*
			Nb				**Ni**				**P**				**Pb**		
V	1	4.5×10^{-5}	4.5×10^{-5}	0.52	0.48	0.01	0.01	0.040	0.84	9.4×10^{4}	9.4×10^{4}	9.39	*0.006*	0.091	0.091	0.082	0.79
S	19	0.019	0.001	9.85	*$<10^{-4}$*	156	8.23	25.7	*$<10^{-4}$*	4.3×10^{6}	2.3×10^{5}	18.9	*$<10^{-4}$*	163	8.60	5.98	*10^{-4}*
V × S	19	0.002	10^{-4}	3.97	*$<10^{-4}$*	6.08	0.32	29.4	*$<10^{-4}$*	2.3×10^{5}	1.2×10^{4}	10.0	*$<10^{-4}$*	27.3	1.44	68.8	*$<10^{-4}$*
			Rb				**S**				**Sb**				**Sc**		
V	1	6.32	6.32	0.67	0.42	3.0×10^{5}	3.0×10^{5}	2.87	0.11	10^{-4}	10^{-4}	0.083	0.77	0.005	0.005	1.06	0.31
S	19	8701	4578	40.9	*$<10^{-4}$*	4.4×10^{6}	2.3×10^{5}	1.94	0.080	0.36	0.019	10.5	*$<10^{-4}$*	0.24	0.013	2.70	*0.018*
V × S	19	213	11.2	15.3	*$<10^{-4}$*	2.3×10^{6}	1.2×10^{5}	4.40	*$<10^{-4}$*	0.034	0.002	25.2	*$<10^{-4}$*	0.089	0.005	0.75	0.76
			Se				**Sn**				**Sr**				**Th**		
V	1	0.008	0.008	1.48	0.24	0.027	0.027	2.21	0.15	0.02	0.02	0.01	0.98	7.3×10^{-7}	7.3×10^{-7}	0.002	0.97
S	19	0.21	0.011	1.83	0.10	3.29	0.17	11.6	*$<10^{-4}$*	1.2×10^{4}	629	13.34	*$<10^{-4}$*	0.11	0.006	12.3	*$<10^{-4}$*
V × S	19	0.11	0.006	2.52	*0.001*	0.28	0.015	16.7	*$<10^{-4}$*	896.3	47.2	103	*$<10^{-4}$*	0.009	*5×10^{-4}*	8.23	*$<10^{-4}$*
			Ti				**Y**				**Zn**				**Zr**		
V	1	19.4	19.4	2.95	0.10	0.083	0.083	1.52	0.23	375	375	5.59	*0.029*	12.2	12.2	2.60	0.12
S	19	1459	76.8	9.73	*$<10^{-4}$*	6.23	0.33	5.12	*4×10^{-4}*	4.3×10^{-4}	2262	27.9	*$<10^{-4}$*	198	10.4	1.83	0.10
V × S	19	150	7.89	13.0	*$<10^{-4}$*	1.22	0.062	49.7	*$<10^{-4}$*	1538	80.9	20.7	*$<10^{-4}$*	109	5.71	77.8	*$<10^{-4}$*

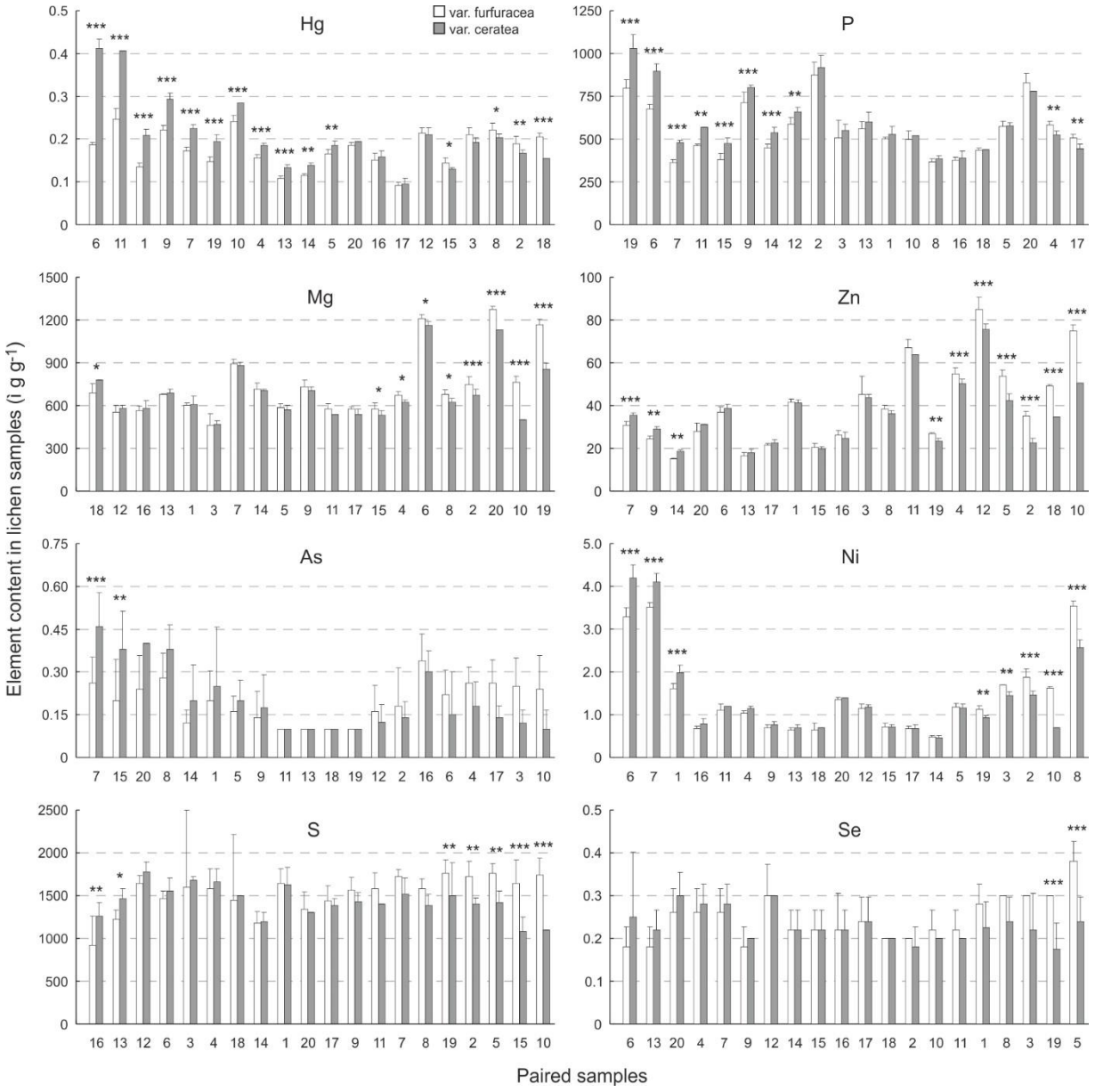

Fig. 4 Element content in paired samples of the two varieties of *P. furfuracea* collected at 20 remote sites. Panels refer to 8 elements out of 40, selected on the basis of the GLMM results of Table 3, whose content in *Pseudevernia furfuracea* was marginally affected by lichen variety (Hg, P, Mg and Zn), significantly affected by sample effect (As, Ni) or not affected by first order main effects (S, Se). In each panel, data refer to mean and 95 % confidence interval of element content in each sample of each variety, calculated over N ≤ 5 technical replicates, and samples are ranked by decreasing inter-varietal difference. Asterisks above bars indicate significant inter-varietal pair-wise differences (*: *P<0.05;* **: *P*<0.01; ***: *P*<0.001, post-hoc LSD test for the interactive effect of sample and variety).

4. Discussion

In this study we showed that the baseline elemental content of *P. furfuracea* in remote Italian montane sites is mostly affected by large-scale, site-dependent factors. Besides such context-dependency, a residual variability of elemental composition at lower spatial scale (i.e. within cluster of sites corresponding to geographical areas homogeneous for lithology and tree species) does exists, but it cannot be ascribed to a systematic effect of variety-related traits. Contrarily, infraspecific differences are highly variable in terms of magnitude and direction, and in some cases do not exceed variation associated to the analytical procedures, as assessed across technical replicates. Our results bear some important implications for biomonitoring with lichen transplants.

4.1 Infraspecific variability

Despite the frequent use of *Pseudevernia furfuracea* in active biomonitoring surveys, the infraspecific variability of element composition was scarcely investigated. Recently, Malaspina et al. (2014) observed a consistent difference for 15 out of 18 tested elements in samples collected in a single site in the Western Alps (Valtournenche, NE Aosta Valley), with a systematically higher content in the nominal variety compared to var. *ceratea*. Differently, our results showed at most marginal inter-varietal differences for only 4 out of the 40 tested elements (i.e. Hg, Mg, P and Zn). When considering element content averaged over all the 20 collection sites, var. *furfuracea* showed higher content than var. *ceratea* only in the case of Zn, apparently consistent with the findings by Malaspina et al. (2014). Conversely, for Hg, Mg and P, the opposite trend was found. Moreover, inter-varietal differences were largely variable, both in terms of sign and magnitude, across different paired samples. This finding excludes a systematic effect of taxonomic-related traits on the element composition of the target species (Fig. 3, Supplementary Table S3).

Malaspina et al. (2014) cautiously hypothesized that inter-varietal differences in element composition, as well as in bioaccumulation capacity, could be due to differences in the lichen substance pools characterizing the two varieties, namely the presence/absence of olivetoric acid, eventually co-occurring with physodic and oxyphysodic acids (Culberson 1969; Elix and Ernst-Russell 1993). However, other factors might be involved as well. Among these, physical traits such as thallus branching, wrinkling and roughness of the external surfaces are known to affect element accumulation in lichens in relation to particle entrapment (Bargagli and Mikhailova 2002; Riga-Karandinos and Karandinos 1998). The external surface of *P. furfuracea* is greatly increased by the development of isidia, i.e. symbiotic propagules which also increase the gas exchange rates (Tretiach et al. 2005) and the water holding capacity (Jahns 1984) of a lichen thallus. The efficient dispersal by isidia (Purvis et al. 1992) makes the presence of such structures a very common trait in *P. furfuracea*, while a considerable morphological variability has been related to specific, different ecological contexts (Rikkinen 1997). Consequently, the baseline elemental composition in densely isidiate thalli, characterized by higher exchange surface per area and mass unit (Tretiach et al. 2005; Bertuzzi and Tretiach 2013), may be enriched compared to co-occurring scarcely isidiate thalli.

A further, interesting consideration, is that 3 out of 4 elements showing overall inter-varietal difference (Table 3), are linked to chlorophyll (Mg, Zn) and to the granules of polyphosphates (P) present in the growing pseudomeristematic apices (Giordani and Brunialti 2002). Given that isidia are pseudomeristematic outgrowths and are particularly rich in algal cells (Tretiach et al. 2005), it could be argued that densely isidiate thalli contain higher levels of Mg, Zn and P compared to scarcely isidiate ones. This could likely occur also in the case of Hg, which accumulates in the plastoglobules of the large chloroplast of the lichen photobiont *Trebouxia* (P. Modenesi, pers. comm.). In this perspective, our results suggests the need of a reliable assessment of isidia abundance per surface and mass unit (e.g. Tretiach et al. 2005), which is certainly a painstaking work, in place of a simple visual assessment.

Additional putative determinants of infraspecific variability in element content could be related to the small-scale location of collected thalli, particularly in relation to the position along tree branches (Adamo et al. 2008). We cannot provide direct evidence about environmental effects at within-site scale in pristine habitats, nor we can infer the effect of morphological traits at single-thallus level,

because our data do not refer to environmental/ biological replicates for either variety at each sampling site. However, our results suggest that micro-environmental conditions and morphological traits, rather than taxonomic differences, should be taken into account in further experimental work, in order to clarify their relative importance.

4.2 Context-dependency of baseline element composition

Our multivariate analysis showed a pattern of element content in lichen samples consistent with variation of potential predictors such as lithology and location, but not with taxonomic variety. Given the exploratory purpose of PCA and CA, the causal role and relative contributions of potential predictors cannot be directly inferred from our results. However, they provide useful indications, further supported by the results of our first GLMM.

We found a correspondence between the CA clusters of samples and the geographical distribution of the collection sites, with significant differences of element content among samples collected in the Eastern and the Western Alps. Samples from the Apennines did not provide consistent results, likely due to the low number of sites. Such distributional pattern was consistent even considering different preliminary sample classifications by CA (data not shown). It obviously reflects differences of environmental conditions among these three macro-areas, indicating that the elemental composition of *P. furfuracea* is mostly affected by large-scale variations of environmental factors. Among these, previous studies on different lichen species indicated major effects of meteoclimatic conditions (Garty 2001), lithology (Agnan et al. 2014, 2015) and altitude (Král et al. 1989; Doucet and Carignan 2001; Bargagli 1998), while evidence on land use is limited to transplanted lichens (Conti et al. 2004; Sorbo et al. 2008). No suitable information about the effects of land use can be derived from our data, which refer to samples collected within the same land cover class (i.e. coniferous forests, Corine land cover class 3.1.2, Bossard et al. 2000). On the other hand, our study provides indication about the relative contributions of lithology and climatic conditions and their possible interactions. CA, based only on element content data, produced a sample classification which is also consistent with the type of lithological substrate occurring at the collection sites. In detail, the samples from the Eastern Alps (cluster I, limestone and sandstone substrates) showed the lowest mean content of all groups of elements (Fig. 3), and the minimum content of all elements, with the exception of Sr (Supplementary Table S3). Major climatic peculiarities of the collection sites from Eastern Alps, compared to other collection sites, could explain the observed pattern. The lowest values of element content were recorded in the Dolomites and Carnic Alps (sites 13-14 and 15-17, respectively, Supplementary Table S3), characterized by higher yearly rainfall and snowfall (Privitera et al. 2010; Marchetti and Panizza 2001) than in western and southern sites. Especially, Carnic Alps are considered a hot spot of precipitation in the Alps at the mesoscale (Cicogna et al. 1996; Frei and Schár 1998; Isotta et al. 2013). Therefore, the observed pattern could be related to a net negative balance of element content in these sites resulting from the positive contributions of element-rich depositions by fogs and the "washing effect" by heavy rains (Knops et al. 1991). According to this hypothesis, climatic-dependent effects would reduce the thallus element content and overcome particle contribution by bedrock weathering and soil erosion. This is supported by the generalized lower element content in rainiest sites, independent of the considered chemical species (Supplementary Table S3), while under the

opposite hypothesis the occurrence of minimum values for specific elements should be expected in different regions according to soil- and lithology-dependent contributions.

Differently, samples from the Southern Apennines (cluster III, intrusive siliceous substrates) showed the highest mean content of the 19 elements of group 3 (Fig. 3), and, by far, the maximum content of almost all of them, including most of the lithogenic ones (e.g. Al, Co, Fe, Li, Th, Ti, Pacyna 1986; Nriagu 1989) and all the rare earth elements considered in our study (i.e. Ce, La, Sc, Y) (Fig. 1a, Supplementary Table S3). Interestingly, such elements are often used as tracers of geochemical transport processes (Tricca et al. 1999; Aubert et al. 2001; Laveuf and Cornu 2009). The lithological substrate of the area is characterized by an igneous-metamorphic complex with small veins of intrusive rocks from felsic to femic (granodiorite, diorite, and gabbro). However, the lithology at collection site is mostly composed by strongly weathered quartzdioritic-granite trondhjemite (intrusive siliceous rocks), with red-brownish coarse sandy soils, prone to erosion (Borrelli et al. 2007; Peronace et al. 2009). In such conditions windblown dust may represent an important source of trace elements to lichens (Bargagli 1995; Bergamaschi et al. 2004). Hence, the high levels of lithogenic and rare earth elements in samples from the Southern Apennines, indicate that particle re-depositions due to bedrock weathering and soil erosion could play a major controlling role on lichen element content in sites characterized by low precipitations.

Some previous observations of lichen elemental content changing with altitude were not clearly explained (e.g. Doucet and Carignan 2001). Other authors reported an increase of Cd and Pb along altitudinal transects, from the base to the top of the mountains (Král et al. 1989; Bargagli 1998), ascribed to long-range transport phenomena (Bargagli 1998). Our results concerning Cd, Pb, or other elements did not show altitudinal patterns, likely because our dataset includes a narrower altitudinal range compared to previous studies.

However, the enrichment of potentially toxic metals in samples from Western Alps could be the product of medium-long-range transport from anthropogenic sources located in the most industrialized and densely populated regions of Italy.

4.3 Implications for biomonitoring

Our findings bear important implications for the use of the target species in active biomonitoring. In particular, we observed a statistically significant effect of the lichen collection site on the baseline content of all elements, with few exceptions (i.e. S, Se and Zr). Although the usual approach to active biomonitoring is to perform analysis based on EC ratios (i.e. ratio of post- to pre-exposure concentration values in bulk samples) lichen samples with different pre-exposure element content, as we observed in different sites, may provide different bioaccumulation results when exposed under the same conditions (Frati et al. 2005). From a methodological point of view, the use of bulk samples should avoid multiple issues related to pre-exposure data variability. In addition, our study confirms the importance of collecting lichen material for transplants in homogeneous ecological conditions, preferably in a single restricted remote area. Surprisingly, such recommendation has not been specifically included in methodological guidelines for active biomonitoring with lichen transplants (e.g. Gailey and Lloyd 1986; Mikhailova 2002), even though in most applications, also due to practical reasons, lichen material was actually collected in a single site (e.g. Adamo et al. 2003, 2007, 2008; Tretiach et al. 2007, 2011a; Nascimbene et al. 2014; Kodnik et al. 2015). In this

respect, our results also suggest for Italy two areas where the material of *P. furfuracea* could be preferentially collected, due to the generalized low content of most elements (Supplementary Table S3). In particular, limited to trace elements of environmental concern, some showed their minima in the surroundings of Passo Fedaia (As, Ba in site 13 and Cd, Cu, Ni, Pb, Sb and Zn in site 14, Table 1), on the northern slope of Mt. Marmolada, the highest peak of the Dolomites. Other elements were lowest in the surroundings of the Sauris lake, in the Carnic Alps (Mn in site 15, Cr, Hg and Ti in site 17, Cs in both sites, Table 1). The levels of Al, Co and Fe were not different in both areas.

Another relevant indication that can be drawn from our results concerns the simultaneous inclusion of both *P. furfuracea* varieties within the transplanted material. We have clearly demonstrated that inter-varietal differences in element composition are mostly negligible and limited to specific elements at within-site scale (Secti. 4.2). The selection of a single variety for transplant purpose has recently been suggested by Malaspina et al. (2014) as a precaution, as already applied by a small number of researchers (Rinino et al. 2005; Tretiach et al. 2007; 2011a). In this respect, a selective collection of the nominal variety is an easy task because below a certain altitude (typically 1200 m a.s.l. in the Eastern Alps) it forms almost pure stands. Our results, however, do not directly support such suggestion. Though we have demonstrated that the *P. furfuracea* varieties from reference areas do not differ in the content of elements, it may be hypothesized that the transplantation to polluted areas may differently affect the synthesis of secondary metabolites in either variety as a consequence of stress, eventually affecting the amount of elements bounded in such conditons. However, no previous studies on this specific topic are available for the target species, and evidences for other species do not fully support such hypothesis. For example, Pawlik-Skowrońska and Bačkor (2011) reported higher content of secondary metabolites in *Hypocenomice scalaris*, *Lepraria* sp. and *Cladonia furcata* in samples exposed to high levels of Zn and Pb compared to control samples, but such compounds (lecanoric, fumarprotocetraric, stictic and constictic acids) do not occur in our target species. On the other hand, Hauck et al. (2013) showed a positive association between the total content of secondary metabolites (physodic, physodalic, 3-hydroxyphysodic acids, atranorin and chloroatranorin) and the total content of Cu, Fe, Mn and Zn in *Hypogymnia physodes*, a species closely related to *P. furfuracea*. Consistently, for the same species, Białońska and Dayan (2005) had reported an increase of physodalic acid content in thalli transplanted to polluted sites, also showing, interestingly, a decrease of physodic acid content. Considering that physodic and physodalic acids are not diagnostic of the *P. furfuracea* taxonomic variety, and that the taxonomically diagnostic olivetoric acid has not yet been specifically investigated in relation to element content, further studies should test the proposed hypothesis. Rather, in order to minimize the variability of baseline element content in the lichen material, attention should be posed towards the material homogeneity, focused more on morphological and positional characteristics of collected thalli, rather than to the taxonomic variety.

5. Conclusions

This study provided for the first time a large-scale outline of baseline element content in *Pseudevernia furfuracea*, one of the most frequently used lichens in active biomonitoring surveys.

Our findings highlighted a remarkable context-dependency of element content variability, likely related to lithology and climatic conditions at the remote collection sites. Data variability was almost unrelated to taxonomic traits at infraspecific level, with inter-varietal differences, in some cases, not overcoming uncertainty associated to the analytical laboratory procedures. Conversely, morphological and positional characteristic of the collected thalli may play a relevant role, a hypothesis which requires further investigation.

Under an application perspective, our study provides important indications relevant for the methodological advance and standardization of active biomonitoring techniques based on a common, not endangered lichen species. In particular, the results of Malaspina et al. (2014) about intervarietal differences in baseline elemental composition and bioaccumulation capacity cannot be confirmed, and a much broader analysis suggested that they are not relevant for active biomonitoring application. The use of morphologically homogeneous material from a single collection area is suggested, regardless of infraspecific taxonomic differences, if large populations are locally available. However, additional investigation should quantitatively assess the role of lichen substances in relation to bioaccumulation phenomena.

Finally, a reference source for lichen material suitable for active biomonitoring application should be operationally defined for a full standardization of the transplant technique.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLES S1-S3

Supplementary Table S1. Number of technical replicates obtained for each lichen variety (c: var. *ceratea*, f: var. *furfuracea*) and site.

Site	Variety		Tot
	c	f	
1	3	5	8
2	4	5	9
3	5	3	8
4	3	5	8
5	5	5	10
6	3	5	8
7	5	4	9
8	4	5	9
9	4	5	9
10	3	5	8
11	3	5	8
12	4	5	9
13	5	5	10
14	3	5	8
15	3	5	8
16	5	5	10
17	5	5	10
18	3	3	6
19	4	5	9
20	3	4	7
Tot	**77**	**94**	**171**

Supplementary Table S2. Detection limit (LOD) of the analytical procedure used at ACME Analytical Laboratories (Vancouver, Canada) for the determination of element concentration in lichen samples by ICP-MS. Expected values, measured mean values, and recovery (mean and 95% confidence interval) of element content in two in-house plant leaves standard reference materials (CDV-1 and V16) and in the standard CRM BCR 482 'lichen' *P. furfuracea*. Confidence limits were calculated on 11 analytical replicates for the internal standards, and on 5 replicates for the lichen standard. For BCR 482, expected values are either certified or indicative values (Quevauviller et al. 1996): the latter are indicated in the table with the "i" apex. All concentration values are expressed in μg g^{-1}.

Element	LOD	CDV-1			V16			BCR 482		
		Expected	Measured	Recovery % (95% C. I.)	Expected	Measured	Recovery % (95% C. I.)	Certified / indicative [i]	Measured	Recovery % (95% C. I.)
Ag	0.002	0.009	0.010	109.1 (97.6÷120.6)	0.032	0.037	115.6 (109.2÷122.1)	-	-	n.a.
Al	100	1500	1509	100.6 (95.1÷106.1)	454	473	104.1 (97.2÷111)	1103	400	36.3 (36.3÷36.3)
As	0.1	1.30	1.26	97.2 (85.1÷109.3)	1.6	1.5	93.8 (85.1÷102.4)	0.85	0.68	80.0 (73.5÷86.3)
Au	0.0002	0.0023	0.0021	91.7 (55.5÷127.9)	0.0009	0.0011	125.3 (96.7÷153.8)	-	-	n.a.
B	1	12.0	11.9	99.2 (94.6÷103.9)	5.0	5.1	101.8 (97.8÷105.9)	-	-	n.a.
Ba	0.1	9.0	9.2	102.1 (99.0÷105.2)	1.90	2.03	106.7 (101.9÷111.5)	14.9[i]	9.7	65.4 (63.7÷65.8)
Be	0.1	-	-	n.a.	-	-	n.a.	-	-	n.a.
Bi	0.02	0.020	0.024	118.2 (77.7÷158.7)	-	-	n.a.	-	-	n.a.
Ca	100	19400	20282	104.5 (101.7÷107.4)	3000	3255	108.5 (105.6÷111.4)	2624[i]	2260	86.1 (81.9÷90.2)
Cd	0.01	0.040	0.037	93.2 (85.3÷101.0)	0.086	0.085	98.3 (90.2÷106.4)	0.56	0.53	94.3 (91.9÷96.5)
Ce	0.01	4.9	5.4	110.2 (106.4÷114.1)	0.100	0.104	103.6 (97.4÷109.8)	-	-	n.a.
Co	0.01	2.00	1.98	99.2 (95.0÷103.5)	1.11	1.06	95.5 (86.2÷104.8)	0.32[i]	0.28	88.8 (86.6÷90.7)
Cr	0.1	12.1	13.5	111.6 (104.6÷118.6)	323	309	95.7 (84÷107.3)	4.12	4.18	101.5 (97.0÷105.9)
Cs	0.005	0.121	0.125	103.2 (97.3÷109.1)	0.036	0.037	103.8 (99.9÷107.6)	-	-	n.a.
Cu	0.01	8.6	8.7	101.2 (97.3÷105.0)	6.69	6.89	103 (96÷109.9)	7.03	6.82	97.0 (91.4÷102.6)
Fe	10	2560	2933	114.6 (109.4÷119.8)	4125	4449	107.9 (95.5÷120.3)	804[i]	784	97.5 (94.7÷99.3)
Ga	0.1	0.50	0.55	110.9 (103.9÷117.9)	0.20	0.14	68.2 (51.2÷85.1)	-	-	n.a.
Ge	0.01	0.030	0.018	60.6 (36.4÷84.8)	0.050	0.055	110.9 (87.4÷134.5)	-	-	n.a.
Hf	0.001	0.046	0.046	100.0 (89.4÷110.6)	0.0060	0.0061	101.5 (72.6÷130.5)	-	-	n.a.
Hg	0.001	0.041	0.044	107.3 (98.9÷115.7)	0.041	0.048	116.9 (108.6÷125.1)	0.48	0.45	92.9 (91.4÷93.8)
In	0.02	-	-	n.a.	-	-	n.a.	-	-	n.a.
K	100	1800	1909	106.1 (102.5÷109.6)	2200	2436	110.7 (109.2÷112.3)	3900[i]	4040	103.6 (100÷105.9)
La	0.01	2.31	2.53	109.5 (107÷112)	0.050	0.038	76.4 (66.3÷86.5)	-	-	n.a.
Li	0.01	0.560	0.599	107 (101.4÷112.5)	0.070	0.066	94.8 (82.5÷107.2)	-	-	n.a.
Mg	10	1200	1256	104.7 (102.6÷106.8)	525	558	106.3 (103.8÷108.9)	-	-	n.a.
Mn	1	385	402	104.3 (102.2÷106.4)	720	720	100.0 (98.2÷101.7)	33.0[i]	30.4	92.1 (87.8÷94.9)
Mo	0.01	0.200	0.207	103.6 (97.8÷109.5)	1.60	1.74	108.6 (94.1÷123.2)	0.85[i]	0.43	50.1 (49.3÷50.8)

Supplementary Table S2 (continued)

Element	LOD	CDV-1			V16			BCR 482		
		Expected	Measured	Recovery % (95% C. I.)	Expected	Measured	Recovery % (95% C. I.)	Certified / indicative [i]	Measured	Recovery % (95% C. I.)
Na	10	52.0	57.3	110.1 (104.1÷116.2)	15.0	13.6	90.9 (68.3÷113.5)	-	-	n.a.
Nb	0.01	0.050	0.053	105.5 (96.8÷114.1)	0.11	0.10	90.9 (76.7÷105.1)	-	-	n.a.
Ni	0.1	6.40	6.75	105.5 (101.2÷109.9)	7.40	7.88	106.5 (93÷120)	2.47	2.20	89.1 (84÷93.3)
P	10	380	397	104.5 (100.5÷108.6)	488	481	98.5 (96.5÷100.6)	690[i]	676	98.0 (93.1÷102.6)
Pb	0.01	1.00	0.99	99.5 (95.3÷103.6)	3.00	2.91	97.1 (94.7÷99.5)	40.9	36.7	89.7 (86.3÷90.4)
Pd	0.002	-	-	n.a.	-	-	n.a.	-	-	n.a.
Pt	0.001	-	-	n.a.	-	-	n.a.	-	-	n.a.
Rb	0.1	2.30	2.56	111.5 (107.7÷115.2)	1.70	1.69	99.5 (96.7÷102.2)	-	-	n.a.
Re	0.001	-	-	n.a.	-	-	n.a.	-	-	n.a.
S	100	1000	1500	150.0 (131.7÷168.3)	177	536	303.0 (267.9÷338.1)	2166[i]	2080	96.0 (85÷100.6)
Sb	0.02	0.030	0.025	84.8 (69.5÷100.2)	0.070	0.059	84.4 (77.7÷91.1)	0.35[i]	0.29	83.4 (77.1÷86.7)
Sc	0.1	0.80	0.91	113.6 (104.9÷122.4)	-	-	n.a.	-	-	n.a.
Se	0.1	0.30	0.22	72.7 (55.9÷89.5)	-	-	n.a.	0.6[i]	0.4	70.0 (60.7÷71.9)
Sn	0.02	0.08	0.10	125.0 (118.5÷131.5)	0.230	0.232	100.8 (88.3÷113.3)	1.31[i]	2.05	156.8 (92.3÷221)
Sr	0.5	112	115	102.9 (100.9÷104.9)	11.20	11.24	100.3 (98.3÷102.4)	-	-	n.a.
Ta	0.001	-	-	n.a.	-	-	n.a.	-	-	n.a.
Te	0.02	-	-	n.a.	-	-	n.a.	-	-	n.a.
Th	0.01	0.61	0.65	105.8 (99.9÷111.8)	-	-	n.a.	-	-	n.a.
Ti	1	30.0	29.9	99.7 (93.9÷105.5)	12.0	10.9	90.9 (87.9÷93.9)	-	-	n.a.
Tl	0.02	-	-	n.a.	-	-	n.a.	-	-	n.a.
U	0.01	0.170	0.166	97.9 (93.1÷102.6)	-	-	n.a.	-	-	n.a.
V	2	4.2	9.3	220.8 (208.2÷233.4)	-	136.5455	n.a.	3.74[i]	3.80	101.6 (86.8÷116)
W	0.1	-	-	n.a.	-	-	n.a.	-	-	n.a.
Y	0.001	1.41	1.55	109.7 (107÷112.4)	0.043	0.050	116.1 (108.1÷124)	-	-	n.a.
Zn	0.1	23.3	22.6	96.9 (94.1÷99.7)	39.2	39.0	99.5 (97.8÷101.3)	100.6	94.6	94.1 (91.2÷95.7)
Zr	0.01	1.20	1.29	107.1 (101.7÷112.6)	0.180	0.178	99.0 (88.9÷109.1)	-	-	n.a.

Supplementary Table S3. Element content in paired samples of two taxonomic varieties of the epiphytic lichen *Pseudevernia furfuracea* from 20 different collection sites. Data refer to mean and 95% confidence interval calculated over $3 \leq N \leq 5$ technical replicates for each sample of both lichen varieties (i.e. var. *furfuracea* and var. *ceratea*, in brackets cluster numbers from Fig. 1). Values multiplied by a scale factor for each element (in brackets) refer to actual content in μg g^{-1}. Minimum and maximum for each element are highlighted in bold and italic, respectively.

Gr	Element	14 (I)	17 (I)	15 (I)	16 (I)	13 (I)	9 (I)	19 (I)	18 (I)	2 (I-II)	10 (I-II)	1 (II)	4 (II)	3 (II)	5 (II)	6 (II)	7 (II)	8 (II)	12 (II)	11 (II)	20 (III)
1	Ag (× 10^{-2})	**1.1 ± 0.1**	1.3 ± 0.1	1.3 ± 0.1	1.5 ± 0.1	1.3 ± 0.1	**1.1 ± 0.1**	1.3 ± 0.2	1.6 ± 0.6	1.4 ± 0.1	3.0 ± 0.3	2.2 ± 0.1	2.6 ± 0.1	2.3 ± 0.2	2.3 ± 0.2	2.2 ± 0.1	2.4 ± 0.2	2.7 ± 0.1	2.9 ± 0.4	*6.0 ± 1.1*	1.5 ± 0.2
	Au (× 10^{-1})	**0.1 ± 0.0**	0.5 ± 0.1	0.5 ± 0.0	0.7 ± 0.1	**0.1 ± 0.0**	0.6 ± 0.1	0.4 ± 0.2	3.2 ± 1.7	0.8 ± 0.1	1.8 ± 0.6	0.6 ± 0.2	1.5 ± 0.8	1.5 ± 0.2	1.0 ± 0.1	3.0 ± 1.3	2.5 ± 0.8	1.7 ± 0.1	1.6 ± 0.2	*5.6 ± 2.0*	1.6 ± 1.3
	Bi (× 10^{-2})	**2.1 ± 0.2**	3.0 ± 0.0	5.3 ± 2.4	3.9 ± 0.5	3.0 ± 0.0	4.1 ± 0.5	2.2 ± 0.3	2.7 ± 1.1	4.6 ± 0.8	7.7 ± 1.9	6.1 ± 0.9	5.6 ± 0.6	6.3 ± 1.0	6.1 ± 1.6	4.0 ± 0	4.8 ± 1.6	4.1 ± 0.5	*9.8 ± 1.3*	7.0 ± 2.0	2.2 ± 0.0
	Cs (× 10^{-1})	2.1 ± 0.1	**0.5 ± 0.0**	**0.5 ± 0.0**	0.6 ± 0.1	0.6 ± 0.0	1.0 ± 0.1	**0.5 ± 0.0**	0.7 ± 0.0	1.2 ± 0.1	1.6 ± 0.1	0.9 ± 0.1	0.9 ± 0.0	0.6 ± 0.1	1.7 ± 0.3	1.0 ± 0.1	0.6 ± 0.1	1.8 ± 0.3	0.9 ± 0.1	*5.7 ± 0.2*	0.9 ± 0.0
	Cu	**2.8 ± 0.4**	3.8 ± 0.1	4.0 ± 0.1	4.3 ± 0.3	3.9 ± 0.3	5.7 ± 0.3	3.8 ± 0.2	5.7 ± 2.6	5.0 ± 0.7	8.6 ± 2.0	5.6 ± 0.2	10.3 ± 0.4	5.6 ± 0.3	6.9 ± 0.6	7.7 ± 1.0	4.1 ± 0.1	7.6 ± 0.3	*13.7 ± 4.1*	7.3 ± 0.2	3.4 ± 0.1
	Hg (× 10^{-1})	1.3 ± 0.1	**0.9 ± 0.1**	1.4 ± 0.1	1.5 ± 0.1	1.2 ± 0.1	2.5 ± 0.3	1.7 ± 0.2	1.9 ± 0.6	1.8 ± 0.1	2.5 ± 0.2	1.7 ± 0.3	1.7 ± 0.1	2.0 ± 0.1	1.8 ± 0.1	2.5 ± 1.0	2.0 ± 0.2	2.1 ± 0.1	2.1 ± 0.1	*2.7 ± 0.7*	1.9 ± 0.1
	Mo (× 10^{-1})	**1.1 ± 0.1**	1.7 ± 0.1	1.8 ± 0.1	1.9 ± 0.1	1.4 ± 0.1	2.4 ± 0.2	**1.1 ± 0.1**	2.1 ± 0.6	2.5 ± 0.4	4.5 ± 1.1	3.0 ± 0.3	3.5 ± 0.2	3.0 ± 0.1	3.8 ± 0.5	4.6 ± 0.2	2.5 ± 0.1	3.4 ± 0.2	*5.6 ± 0.2*	3.7 ± 0.1	**1.1 ± 0.1**
	Pb	**1.2 ± 0.1**	2.2 ± 0.1	1.8 ± 0.1	2.3 ± 0.1	1.9 ± 0.1	2.0 ± 0.3	2.4 ± 0.1	3.0 ± 0.2	2.4 ± 0.4	3.8 ± 0.4	3.4 ± 0.7	3.9 ± 0.3	*5.5 ± 0.8*	3.9 ± 0.3	2.4 ± 0.1	2.4 ± 0.4	2.3 ± 0.1	3.7 ± 0.3	3.9 ± 0.8	2.6 ± 0.2
	Rb (× 10^{1})	1.8 ± 0.1	**0.4 ± 0.0**	**0.4 ± 0.0**	0.6 ± 0.0	0.7 ± 0.0	1.7 ± 0.1	0.7 ± 0.0	1.4 ± 0.2	2.5 ± 0.1	1.5 ± 0.0	1.0 ± 0.1	2.1 ± 0.1	0.8 ± 0.1	2.6 ± 0.2	1.1 ± 0.1	0.8 ± 0.1	1.5 ± 0.1	1.2 ± 0.2	*3.4 ± 0.3*	0.7 ± 0.0
	Sb (× 10^{-1})	**0.3 ± 0.1**	0.5 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.8 ± 0.2	0.5 ± 0.1	0.5 ± 0.2	1.1 ± 0.2	1.5 ± 0.3	1.5 ± 0.3	1.2 ± 0.1	1.5 ± 0.2	1.3 ± 0.1	1.2 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	*2.0 ± 0.1*	1.2 ± 0.2	0.4 ± 0.0
	Sn (× 10^{-1})	**1.5 ± 0.1**	2.9 ± 0.2	2.7 ± 0.2	2.8 ± 0.2	2.6 ± 0.1	3.1 ± 0.2	2.2 ± 0.2	1.9 ± 0.7	3.9 ± 0.6	6.7 ± 1.9	4.6 ± 0.5	3.8 ± 0.2	4.5 ± 0.5	4.0 ± 0.4	3.9 ± 0.3	2.4 ± 0.1	3.1 ± 0.1	*7.9 ± 0.5*	4.9 ± 0.5	**1.5 ± 0.1**
	Zn (× 10^{1})	**1.7 ± 0.1**	2.2 ± 0.1	2.0 ± 0.1	2.5 ± 0.1	**1.7 ± 0.1**	2.7 ± 0.2	2.5 ± 0.2	4.4 ± 1.5	2.9 ± 0.5	7.1 ± 1.0	4.1 ± 0.1	5.3 ± 0.2	4.4 ± 0.2	4.8 ± 0.5	3.7 ± 0.1	3.3 ± 0.2	3.7 ± 0.1	*8.1 ± 0.5*	6.6 ± 0.2	2.8 ± 0.2
2	As (× 10^{-1})	1.6 ± 0.7	2.0 ± 0.8	2.9 ± 1.1	3.2 ± 0.6	**1.0 ± 0.0**	1.6 ± 0.7	**1.0 ± 0.0**	**1.0 ± 0.0**	1.6 ± 0.7	2.2 ± 1.2	2.2 ± 1.0	2.2 ± 0.6	1.6 ± 1.0	1.8 ± 0.4	2.0 ± 0.7	*3.6 ± 1.0*	3.3 ± 0.6	1.4 ± 0.5	1.0 ± 0.0	2.7 ± 1.2
	Cr	2.3 ± 0.1	**2.2 ± 0.1**	2.4 ± 0.2	2.5 ± 0.1	2.6 ± 0.1	2.6 ± 0.2	2.4 ± 0.2	**2.2 ± 0.2**	2.9 ± 0.3	2.6 ± 0.2	3.2 ± 0.3	2.6 ± 0.1	2.8 ± 0.2	2.5 ± 0.1	*5.2 ± 0.6*	3.6 ± 0.2	3.5 ± 0.2	2.4 ± 0.1	2.6 ± 0.1	3.2 ± 0.2
	Ge (× 10^{-2})	1.9 ± 0.5	1.2 ± 0.3	1.4 ± 0.4	1.1 ± 0.2	1.1 ± 0.2	1.3 ± 0.4	1.1 ± 0.2	**1.0 ± 0.0**	1.1 ± 0.2	**1.0 ± 0.0**	1.1 ± 0.2	1.6 ± 0.6	1.3 ± 0.4	*2.1 ± 0.4*	1.1 ± 0.4	1.2 ± 0.3	1.2 ± 0.3	**1.0 ± 0.0**	**1.0 ± 0.0**	1.8 ± 0.4
	K (× 10^{3})	3.0 ± 0.1	**2.7 ± 0.1**	2.8 ± 0.1	**2.7 ± 0.1**	3.3 ± 0.1	3.8 ± 0.2	3.8 ± 0.2	3.3 ± 0.2	4.0 ± 0.2	3.7 ± 0.4	2.9 ± 0.2	3.4 ± 0.2	**2.7 ± 0.3**	3.4 ± 0.1	*4.1 ± 0.2*	3.3 ± 0.1	**2.7 ± 0.1**	3.6 ± 0.2	3.4 ± 0.1	3.6 ± 0.1
	Ni	**0.5 ± 0.0**	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	1.0 ± 0.1	0.7 ± 0.2	1.7 ± 0.2	1.5 ± 0.4	1.8 ± 0.2	1.1 ± 0.1	1.5 ± 0.1	1.2 ± 0.1	3.5 ± 0.4	*3.8 ± 0.2*	3.1 ± 0.4	1.2 ± 0.1	1.1 ± 0.1	1.4 ± 0.1
	P (× 10^{2})	4.9 ± 0.4	4.7 ± 0.3	4.3 ± 0.4	**3.8 ± 0.2**	5.8 ± 0.2	7.5 ± 0.5	9.0 ± 1.1	4.4 ± 0.2	9.0 ± 0.5	5.0 ± 0.2	5.1 ± 0.2	5.5 ± 0.3	5.4 ± 0.4	5.8 ± 0.1	7.4 ± 1.0	4.2 ± 0.4	**3.8 ± 0.1**	6.2 ± 0.4	4.8 ± 0.5	*9.2 ± 0.3*
	S (× 10^{3})	1.2 ± 0.1	1.4 ± 0.1	1.4 ± 0.3	**1.1 ± 0.2**	1.3 ± 0.1	1.5 ± 0.1	1.6 ± 0.2	1.5 ± 0.6	1.6 ± 0.1	1.6 ± 0.3	1.6 ± 0.2	1.6 ± 0.1	*1.7 ± 0.2*	1.6 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	*1.7 ± 0.1*	1.6 ± 0.2	1.3 ± 0.2
	Sc (× 10^{-1})	3.3 ± 0.8	3.1 ± 0.6	3.4 ± 0.6	**2.7 ± 0.6**	3.9 ± 0.5	3.1 ± 0.8	3.0 ± 0.5	**2.7 ± 1.1**	3.4 ± 0.6	3.5 ± 0.5	3.4 ± 0.4	3.4 ± 0.5	3.3 ± 0.4	3.1 ± 0.5	3.4 ± 0.7	3.9 ± 0.4	3.3 ± 0.5	**2.7 ± 0.4**	3.3 ± 1.2	*4.8 ± 0.8*
	Se (× 10^{-1})	2.2 ± 0.3	2.4 ± 0.4	2.2 ± 0.3	2.2 ± 0.4	2.0 ± 0.4	**1.9 ± 0.2**	2.4 ± 0.5	2.0 ± 0.0	**1.9 ± 0.2**	2.2 ± 0.4	2.6 ± 0.4	2.7 ± 0.4	2.4 ± 0.7	*3.1 ± 0.6*	2.0 ± 0.5	2.7 ± 0.4	2.7 ± 0.4	3.0 ± 0.4	2.2 ± 0.4	2.7 ± 0.5
3	Al (× 10^{2})	**2.0 ± 0.0**	**2.0 ± 0.0**	2.6 ± 0.4	2.9 ± 0.2	3.6 ± 0.4	3.0 ± 0.0	4.1 ± 0.5	3.0 ± 0.0	3.8 ± 0.6	2.8 ± 0.4	3.9 ± 0.5	3.6 ± 0.4	3.7 ± 0.4	2.9 ± 0.2	4.0 ± 0.0	4.0 ± 0.0	4.1 ± 0.2	3.0 ± 0.0	3.2 ± 0.4	*9.7 ± 0.5*
	Ba (× 10^{1})	0.7 ± 0.1	1.0 ± 0.0	0.7 ± 0.1	0.8 ± 0.1	**0.6 ± 0.0**	**0.6 ± 0.0**	2.0 ± 0.4	**0.6 ± 0.0**	1.0 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.0 ± 0.0	1.1 ± 0.1	0.8 ± 0.1	1.1 ± 0.0	1.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	1.2 ± 0.2	*2.9 ± 0.1*
	Ca (× 10^{3})	3.5 ± 0.3	2.6 ± 0.2	**2.3 ± 0.2**	3.8 ± 0.3	3.4 ± 0.3	5.7 ± 1.1	9.5 ± 0.8	6.6 ± 0.6	4.4 ± 0.9	4.9 ± 0.9	5.2 ± 0.2	5.8 ± 0.3	5.2 ± 0.4	5.1 ± 0.4	5.2 ± 0.2	4.8 ± 0.5	2.4 ± 0.2	3.9 ± 0.3	3.8 ± 0.4	*19.9 ± 2.2*
	Cd (× 10^{-1})	**0.7 ± 0.0**	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	1.0 ± 0.2	1.5 ± 0.2	1.2 ± 0.6	1.0 ± 0.1	1.7 ± 0.1	1.1 ± 0.1	1.4 ± 0.1	1.7 ± 0.3	1.5 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	1.6 ± 0.1	1.9 ± 0.1	1.6 ± 0.2	*3.1 ± 0.6*
	Ce	**0.5 ± 0.0**	**0.5 ± 0.1**	0.6 ± 0.0	0.6 ± 0.1	0.8 ± 0.1	0.6 ± 0.0	0.7 ± 0.2	0.7 ± 0.0	0.8 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	0.9 ± 0.0	0.7 ± 0.1	0.6 ± 0.1	*2.3 ± 0.1*
	Co	**0.1 ± 0.0**	0.1 ± 0.0	**0.1 ± 0.0**	**0.1 ± 0.0**	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	*0.5 ± 0.0*
	Fe (× 10^{3})	**0.2 ± 0.0**	**0.2 ± 0.0**	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.4 ± 0.0	0.3 ± 0.1	*1.1 ± 0.0*
	Hf (× 10^{-2})	2.5 ± 0.5	2.5 ± 0.9	**2.4 ± 0.3**	3.0 ± 0.4	3.1 ± 0.9	3.4 ± 0.3	5.6 ± 2.3	10.6 ± 8.5	4.4 ± 1.1	4.2 ± 2.3	5.0 ± 0.6	5.9 ± 1.1	6.0 ± 3.2	4.2 ± 0.4	7.1 ± 2.1	4.5 ± 1.1	5.3 ± 2.0	**2.4 ± 0.4**	4.1 ± 4.0	*13.1 ± 5.0*
	La (× 10^{-1})	**1.9 ± 0.1**	**1.9 ± 0.1**	2.3 ± 0.1	2.7 ± 0.2	3.1 ± 0.2	2.6 ± 0.2	3.1 ± 0.6	2.7 ± 0.2	3.5 ± 0.5	2.9 ± 0.6	4.2 ± 0.5	3.9 ± 0.3	4.3 ± 0.3	3.0 ± 0.3	2.8 ± 0.3	3.8 ± 0.3	3.9 ± 0.2	2.8 ± 0.2	2.7 ± 0.2	*9.6 ± 0.4*
	Li (× 10^{-1})	**1.3 ± 0.1**	**1.3 ± 0.1**	1.8 ± 0.1	1.9 ± 0.2	2.3 ± 0.1	2.2 ± 0.2	3.0 ± 0.2	2.1 ± 0.4	3.3 ± 0.5	2.5 ± 0.4	3.2 ± 0.3	3.3 ± 0.3	3.1 ± 0.3	2.3 ± 0.1	3.1 ± 0.5	2.7 ± 0.3	3.2 ± 0.1	2.5 ± 0.3	1.9 ± 0.3	*6.9 ± 0.2*
	Mg (× 10^{3})	0.7 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	**0.5 ± 0.0**	0.7 ± 0.0	0.7 ± 0.0	1.0 ± 0.2	0.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.0	0.6 ± 0.0	**0.5 ± 0.0**	0.6 ± 0.0	*1.2 ± 0.0*	0.9 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	*1.2 ± 0.1*
	Mn (× 10^{2})	0.8 ± 0.1	0.9 ± 0.1	**0.3 ± 0.0**	0.5 ± 0.0	0.5 ± 0.1	0.6 ± 0.0	0.9 ± 0.1	**0.3 ± 0.2**	0.7 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	1.0 ± 0.0	0.5 ± 0.0	0.5 ± 0.1	0.9 ± 0.1	0.9 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.1	*1.8 ± 0.1*
	Na (× 10^{2})	0.6 ± 0.1	0.4 ± 0.0	0.3 ± 0.1	**0.2 ± 0.0**	0.5 ± 0.0	0.4 ± 0.1	1.1 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	1.3 ± 0.3	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	**0.2 ± 0.0**	*1.4 ± 0.1*
	Nb (× 10^{-2})	**1.6 ± 0.4**	1.2 ± 0.3	2.0 ± 0.4	2.0 ± 0.4	2.6 ± 0.4	2.0 ± 0.4	3.2 ± 0.5	1.7 ± 1.1	2.8 ± 0.6	3.0 ± 0.6	3.3 ± 0.5	2.7 ± 0.4	3.4 ± 0.4	3.1 ± 0.4	3.7 ± 0.4	3.5 ± 0.6	3.4 ± 0.4	3.1 ± 0.5	2.2 ± 0.4	*8.0 ± 0.6*
	Sr (× 10^{1})	0.9 ± 0.1	0.6 ± 0.0	1.0 ± 0.1	1.3 ± 0.1	0.9 ± 0.1	1.0 ± 0.2	2.8 ± 0.7	1.1 ± 0.0	1.1 ± 0.2	0.7 ± 0.1	0.9 ± 0.1	1.0 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	0.6 ± 0.0	**0.5 ± 0.1**	0.7 ± 0.1	*5.8 ± 0.5*
	Th (× 10^{-1})	**0.4 ± 0.1**	**0.4 ± 0.1**	0.5 ± 0.0	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.0	0.6 ± 0.1	0.5 ± 0.2	0.9 ± 0.1	0.6 ± 0.2	1.1 ± 0.2	0.9 ± 0.1	1.2 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	*1.7 ± 0.1*
	Ti (× 10^{1})	**0.5 ± 0.1**	**0.5 ± 0.0**	0.6 ± 0.0	0.6 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	1.1 ± 0.1	0.6 ± 0.2	1.1 ± 0.1	0.9 ± 0.1	1.2 ± 0.2	0.8 ± 0.1	1.1 ± 0.1	0.8 ± 0.1	1.4 ± 0.1	1.3 ± 0.2	1.1 ± 0.1	0.9 ± 0.1	0.8 ± 0.3	*2.0 ± 0.1*
	Y (× 10^{-1})	**2.5 ± 0.1**	2.8 ± 0.1	2.8 ± 0.1	3.6 ± 0.2	3.2 ± 0.1	3.4 ± 0.2	3.8 ± 0.5	5.1 ± 3.1	4.4 ± 0.8	3.2 ± 0.3	5.6 ± 0.3	6.1 ± 1.1	5.9 ± 1.7	3.8 ± 0.4	5.0 ± 0.5	4.8 ± 0.3	4.5 ± 0.4	2.7 ± 0.3	3.6 ± 2.1	*12.9 ± 2.8*
	Zr	**1.0 ± 0.1**	1.1 ± 0.1	**1.0 ± 0.1**	1.3 ± 0.1	1.2 ± 0.1	1.5 ± 0.2	2.4 ± 1.1	3.6 ± 3.3	1.7 ± 0.4	1.7 ± 1.3	2.2 ± 0.3	2.8 ± 0.8	2.4 ± 1.3	1.8 ± 0.2	3.5 ± 0.6	1.9 ± 0.2	2.4 ± 0.7	**1.0 ± 0.1**	1.7 ± 1.6	*5.3 ± 2.5*

SUPPLEMENTARY FIGURE S1

Supplementary Fig. S1. Map of Italy with indication of the sampling sites of the two varieties of *Pseudevernia furfuracea*. Site numbers as in Table 1 in main text.

Background element content of the lichen *Pseudevernia furfuracea*: A supra-national state of art implemented by novel field data from Italy

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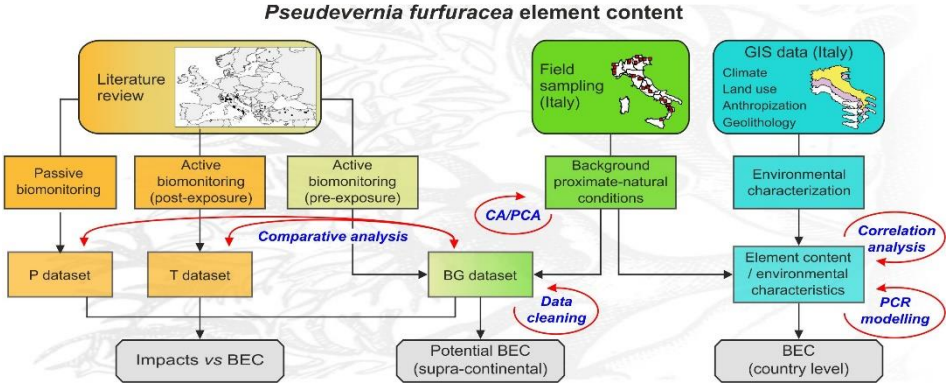
Highlights

• Background element content (BEC) values are needed to assess pollution levels.
• *Pseudevernia furfuracea* is a commonly used biomonitor, whose BECs were unknown.
• BEC values are proposed, based on literature and field data from remote sites.
• Literature data are affected by huge methodological variability.
• Lichen BECs are highly responsive to land use, climate and lithology.

Abstract

In biomonitoring, the knowledge of background element content (BEC) values is an essential pre-requisite for the correct assessment of pollution levels. Here, we estimated the BEC values of a highly performing biomonitor, the epiphytic lichen *Pseudevernia furfuracea*, by means of a careful review of literature data, integrated by an extensive field survey. Methodologically homogeneous element content datasets, reflecting different exposure conditions across European and extra-European countries, were compiled and comparatively analysed. Element content in samples collected in remote areas was compared to that of potentially enriched samples, testing differences between medians for 25 elements. This analysis confirmed that the former samples were substantially unaffected by anthropogenic contributions, and their metrics were therefore proposed as a first overview at supra-national background level. We also showed that bioaccumulation studies suffer a huge methodological variability. Limited to original field data, we investigated the background variability of 43 elements in 62 remote Italian sites, characterized in GIS environment for anthropization, land use, climate and lithology at different scale resolution. The relationships between selected environmental descriptors and BEC was tested using Principal Component Regression (PCR) modelling. Elemental composition resulted significantly dependent on land use, climate and lithology. In the case of lithogenic elements, regression models correctly reproduced the lichen content throughout the country at randomly selected sites. Further descriptors should be identified only for As, Co, and V. Through a multivariate approach we also identified three geographically homogeneous macro-regions for which specific BECs were provided for use as reference in biomonitoring applications.



Keywords: air pollution; baseline; bioaccumulation; particulate matter; *Pseudevernia furfuracea*

1. Introduction

Lichens are widely used as bioaccumulators (Herzig et al. 1989; Garty 2001) to monitor deposition of airborne persistent pollutants (Brunialti and Frati 2014), because their pollutant content is significantly related to the bulk atmospheric depositions (e.g. Herzig et al. 1989; van Dobben et al. 2001). In bioaccumulation studies, a key issue is the interpretation of the pollutant contents in terms of deviation from a pre-existing condition, often corresponding to unaltered, "natural" references. Several approaches have been suggested to quantitatively assess such deviation: (*i*) interpretative scales, i.e. ranks of increasing alteration matched to corresponding element concentration ranges, based on meta-analysis of available data of different species (Nimis and Bargagli 1999) or single species (Nimis et al. 2001; Tretiach and Baruffo 2001a); (*ii*) "Exposed-to-Control ratio" (EC ratio), limited to transplant applications, as the element concentration ratio in exposed to unexposed samples (Frati et al. 2005); (*iii*) comparison with "background values", i.e. baseline element concentration values measured in samples collected in remote areas, far distant from known emission sources (Bargagli 1998). In this framework, the knowledge of background content of persistent chemicals is of primary importance for the evaluation of pollution phenomena, in several ecological compartments (Reimann and Garrett 2005).

While chemical backgrounds are frequently reported for soils and sediments (e.g. Carral et al. 1995; Chen et al. 1999; Rodríguez et al. 2006), biological matrices have been less investigated, with exceptions regarding intertidal organisms (Carral et al. 1995), mosses and vascular plants (Markert and De Li 1991; Chiarenzelli et al. 2001). In the case of lichens, background element content (BEC) values were reported for pools of epiphytic foliose or fruticose species (Bargagli 1998) and for a single species (*Hypogymnia physodes*: Bennet 2000) based on literature reviews. Values of BEC derived from *ad hoc* designed field campaigns were reported for pooled epiphytic species collected in different mountain systems of the world (Bergamaschi et al. 2004), for the epilithic *Umbilicaria decussata* in Antarctica (Bargagli et al. 1999), and for two species of *Nephroma* and *Usnea* from Patagonia (Monaci et al. 2012). Several criticalities affect current available lichen BEC values: (a) data pooled for different taxa are problematic (Djingova et al. 2004), since the species may accumulate differently (Nimis et al. 2001; Tretiach and Baruffo 2001b; Minganti et al. 2003); (b) the selected species are not standard biomonitors; (c) previous reviews did not critically consider methodological differences in sample pre-processing (e.g. washing, drying) and analytical procedures (e.g. acid digestion procedure) among data sources (Adamo et al. 2008; Baffi et al. 2002); (d) even when based on purposed field survey, the fairly low number of sites in single remote areas does not ensure the representativeness of the overall element background variation in the target macro-region. Furthermore, (e) element composition in remote areas predominantly reflects local environmental conditions such as lithology, climate and their possible interactions (Incerti et al. 2017), and therefore reliable BEC values should be proposed for homogenous contexts (Matschullat et al. 2000).

In order to overcome such issues, here we assessed the BEC values of the epiphytic lichen *Pseudevernia furfuracea* (L.) Zopf, selected because it is one of the best performing biomonitor of airborne persistent air pollutants used in both active and passive biomonitoring surveys throughout European and extra-European countries (for details, see Supplementary Material, Sect. S1.1). We

surveyed the literature to compile and comparatively analyse methodologically homogeneous datasets encompassing a supra-national spatial scale. Moreover, we integrated the literature data with an extensive field survey and a climatic, lithological, and land use characterization of the collection sites, thus providing BEC data representative of different environmental contexts.

Specific aims were threefold: (*i*) to provide supra-national state of art on BEC values in the target species; (*ii*) to explore BEC pattern at national level, in relation to anthropization, land use, climate and lithological variables, assessed by a GIS-based environmental characterization of the sampling sites; (*iii*) to test the reproducibility of BEC in *P. furfuracea* by multiple regressive models based on target environmental descriptors.

2. Materials and methods

2.1 Literature survey data

Literature search engines Scopus, Google Scholar and "Recent Literature on Lichens" (Culberson et al. 2015) were queried for eligible active and passive biomonitoring studies reporting element contents in *Pseudevernia furfuracea*. Details on search methods and parameters, data gathering and ancillary information are reported in Supplementary Material, Sect. S1.2.

2.2 Field data and environmental characterization of field sites

Thalli of *P. furfuracea* without distinction of the two varieties (Incerti et al. 2017) were collected at 62 remote sites of the main Italian mountain ranges (Supplementary Table S1). Selection of sites, sample pre-processing, chemical analyses, and quality assessment procedures followed Incerti et al. (2017). The collection sites were characterized in terms of anthropization (population density, built-up area cover), land use (occurrence of artificial surfaces, agricultural areas, and forest and semi-natural areas), climate (precipitation and temperature), and lithology (occurrence of igneous, metamorphic, sedimentary carbonate and sedimentary clastic rocks), using thematic maps in a GIS environment, as reported in Supplementary Material, Sect. S1.3.

2.3 Element content datasets

Three different datasets (BG, T, P) were built up. Dataset BG ("background") consisted of our field data merged with pre-exposure control data of active biomonitoring studies, under the assumption that in both cases lichen thalli were purposely collected far from known anthropogenic emission sources. The selected studies had to be methodologically consistent, and the data were carefully cleaned, as detailed in Supplementary Material, Sect. S1.4.

In order to compare the extent of element enrichment in *P. furfuracea* in polluted conditions, the data of post-exposure samples of the same active biomonitoring studies were included into a dataset named *'transplant'* (T), whereas data from biomonitoring studies referring to native thalli collected in differently polluted areas were included into a dataset named *'passive'* (P).

2.4 Data analysis

The determination of background values is a complex task and different methods can be found in the literature (Matschullat et al. 2000). Therefore, following Reimann et al. (2005), we provided a complete series of basic descriptive statistics of element content, separately calculated for each element in the BG dataset, after removal of outliers as reported in Supplementary Material, Sect. S1.4. In detail, mean, standard deviation, median, median absolute deviation (MAD), and 98th percentile were calculated for each element. In order to test possible enrichment in BG samples, BG medians were tested for significant differences compared to T and P medians using Mann-Whitney's U test, considering either all data pooled within each dataset or separately for different land use types.

Limited to field data, the matrix of collection sites × element content, with data standardized for each element, was submitted to Principal Component Analysis (PCA) and Cluster Analysis (CA) using Euclidean distance as distance measure and Ward's method as grouping algorithm. For the resulting clusters of sites, the same descriptive statistics provided for the BG dataset were calculated for each element. Significant environmental differences among clusters of sites were tested using Kruskal-Wallis ANOVA and non-parametric Dunn's post hoc test, for 13 environmental variables preliminarily selected as potential predictors of lichen element content (Supplementary Material, Sect. S1.3).

A Principal Component Regression (PCR) model was fitted for each element to assess the relationships between environmental variables and lichen BEC, while avoiding possible collinearity among the predictors. First, a matrix of collection sites × environmental variables was submitted to PCA. Then, a multiple linear regression model was fitted for each element, in which the Principal Components (PCs) were considered as independent variables (Jolliffe 2002). PCR models were fitted on data from 40 randomly selected sites (i.e. fitting dataset), and tested on the remaining 22 sites (i.e. validation dataset).

All data analyses and graphics were performed with the software package Statistica v. 10 (StatSoft Inc., Tulsa, OK). Statistical significance was tested at $\alpha = 0.05$ in all cases.

3. Results

3.1 Literature survey on *Pseudevernia furfuracea* element content

The literature search produced 62 studies of active (70%) and passive (29%) biomonitoring, plus 1 methodological paper, carried out in 14 European and 2 non-European countries (Fig. 1; Supplementary Table S2). Expectedly, studies were widely variable in terms of targeted elements, type of biomonitoring application, and lichen exposure conditions as related to specific objectives (Supplementary Fig. S1), as well as with unequal representation of countries (Fig. 1). Less obvious was the remarkable variability of methods detected for pre-treatment of lichen material, acid digestion protocols, analytical techniques and quality assurance/control (Table 1). Moreover, important methodological information was often missing, with 52% and 31% of the studies even failing to report QC methods and digestion protocols, respectively (Table 1, Supplementary Fig. S1).

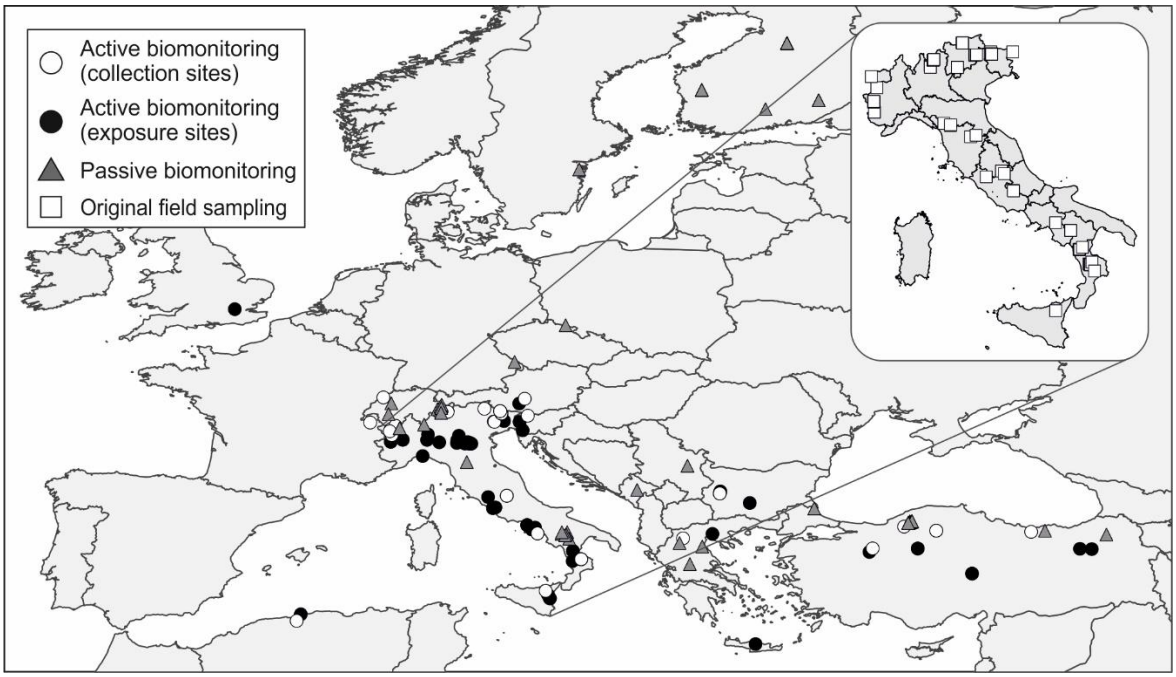


Figure 1. Map of *Pseudevernia furfuracea* collection and exposure sites, symbolized according to source study type.

Table 1. Methodological steps applied in the 62 surveyed studies to assess element content in lichen samples, with types of technical procedures and percent frequency of application.

Sample pre-processing	Acid mixture for sample digestion	Analytical technique for element content determination	QC procedures
Not reported (3%)	Not reported (31%)	Not reported (4.8%)	I Not reported (52%)
Debris removal (56%)	Partial digestion (42%)	Atomic absorption spectrometry: CVAAS, ETAAS, FAAS, GAAS, ZETAAS (33.3%)	II CRM used, but neither CRM type nor recovery percentages specified (6%)
Washing (21%)	– HNO_3	Mass emission spectrometry: ICP-MS (29.6%)	III CRM type specified, but no information reported about recovery (21%)
Oven-drying (11%)	– HNO_3-H_2O_2	X-ray fluorescence: XRF (11.8%)	IV CRM type specified and satisfactory quality of recovery data generically claimed (6%)
Washing + oven-drying (5%)	– HNO_3-HCl	Optical emission spectrometry: ICP-OES (5.6%)	V CRM type specified, and range of recovery percentages reported (5%)
Other (3%)	– HNO_3-H_2O_2-HCl	Atomic emission spectrometry: AES (4.3%)	VI CRM type specified and descriptive statistics of recovery percentages reported for each element (10%).
	– HNO_3-$HClO_4$-HCl	Instrumental neutron activation analysis: INAA (3.8%)	
	– HNO_3-$HClO_4$	Flow injection mercury system: FIMS (1.9%)	
	– HNO_3-$HClO_4$-H_2SO_4	Flash combustion elemental analyser (1.6%)	
	Total digestion (27%)	Isotope-excited X-ray spectrometry (1.6%)	
	– HNO_3-H_2O_2-HF	γ-ray Spectrometry (1.6%)	
	– HNO_3-HCl-HF		
	– HNO_3-HF		

3.2 Element content in background and exposure conditions

The three datasets BG, P and T contained different sets of elements, also showing very different record counts. Overall, dataset BG included 2950 data for 43 elements, dataset P included 513 data for 44 elements, and dataset T included 3760 data for 43 elements (Supplementary Table S3). Expectedly, BG samples showed significantly lower median element content than P and T samples for 10 out of 12, and 21 out of 24 tested elements, respectively (Table 2). When these elements were ranked according to the ratio of median values of T to BG datasets, Na showed the highest value (14.4), followed by Pb (6.1), and by terrigenous elements such as Ti and Al (4.8 and 3.4, respectively). All the other elements ranged between 1.5 and 3, with the exception of Hg (1.1), although with significant BG vs T difference. Non-significant differences between BG and P or T

samples were limited to Mn, K and Se, whereas, in the case of S, median content of BG samples was lower than in T samples, but higher than in P ones.

When stratified by land use, results of the comparative analysis among the three datasets confirmed the general pattern, with T and P samples exposed to rural, urban or industrial conditions showing consistently higher element content compared to BG conditions, although with some exceptions (Fig. 2). In particular, samples of dataset P showed not significantly different content of Cu and Zn at industrialized sites, and of Fe at urbanized sites, compared to BG samples, as also observed for Hg in T samples at urban and industrial sites (Fig. 2).

Table 2. Element content (μg g^{-1}) of the epiphytic lichen *Pseudevernia furfuracea* in the dataset BG ('background'), P ('passive') and T ('transplant'). Data refer to descriptive statistics (counts, mean ± standard deviation, median, median absolute deviation and interquartile range for BG data; counts, median and interquartile range for the elements with data count ≥ 10 in either dataset P or T). For each element, results of statistical testing for differences from the BG data are also reported (Mann-Whitney U test for independent samples; M-W, significant *p*-values in italic).

	BG					P			M-W (BG vs P)	T			M-W (BG vs T)
	n	**Mean ± SD**	**Median**	**MAD**	**IQR**	**n**	**Median**	**IQR**	***p*-value**	**n**	**Median**	**IQR**	***p*-value**
Al	81	457 ± 236	380	90	300 ÷ 535					208	1274	847 ÷ 1710	*< 10^{-10}*
As	63	0.205 ± 0.096	0.180	0.068	0.130 ÷ 0.270	10	0.435	0.330 ÷ 0.500	*3.7·10^{-5}*	145	0.480	0.370 ÷ 0.830	*< 10^{-10}*
Ba	63	12.0 ± 5.5	11.0	2.7	8.1 ÷ 13.6					29	21.9	14.5 ÷ 28.8	*6.8· 10^{-7}*
Ca	74	7615 ± 4092	6185	2416	4680 ÷ 10000					97	15870	10315 ÷ 23310	*< 10^{-10}*
Cd	87	0.183 ± 0.088	0.160	0.050	0.120 ÷ 0.240	23	0.618	0.400 ÷ 0.706	*3.4·10^{-10}*	272	0.330	0.230 ÷ 0.556	*< 10^{-10}*
Co	65	0.255 ± 0.094	0.240	0.070	0.170 ÷ 0.310					110	0.59	0.48 ÷ 0.73	*< 10^{-10}*
Cr	80	2.73 ± 0.77	2.69	0.36	2.43 ÷ 3.12	48	3.44	2.73 ÷ 15.00	*6.9·10^{-6}*	263	4.16	2.73 ÷ 6.00	*1.4·10^{-10}*
Cu	91	5.40 ± 2.09	4.99	1.25	3.78 ÷ 6.63	40	6.60	4.47 ÷ 22.00	*0.002*	329	11.00	6.33 ÷ 22.50	*< 10^{-10}*
Fe	79	516 ± 251	480	132	348 ÷ 620	57	965	612 ÷ 1560	*5.7·10^{-9}*	204	868	630 ÷ 1333	*< 10^{-10}*
Hg	74	0.199 ± 0.059	0.180	0.043	0.160 ÷ 0.250					59	0.200	0.170 ÷ 0.290	*0.043*
K	74	3305 ± 616	3258	442	2867 ÷ 3740					91	3417	2370 ÷ 4540	0.733
Mg	72	766 ± 171	725	96	642 ÷ 847					91	1185	895 ÷ 1819	*< 10^{-10}*
Mn	90	56.5 ± 30.8	50.4	18.5	34.2 ÷ 74.3	43	41.7	26.0 ÷ 71.9	0.269	300	50.0	32.7 ÷ 74.1	0.964
Mo	65	0.249 ± 0.143	0.200	0.082	0.130 ÷ 0.340					91	0.620	0.270 ÷ 1.664	*< 10^{-10}*
Na	73	77.3 ± 67.4	40.0	16.0	30.0 ÷ 134.0					33	575	300 ÷ 918	*< 10^{-10}*
Ni	87	1.72 ± 0.90	1.42	0.51	1.03 ÷ 2.18	38	3.55	1.97 ÷ 20.00	*4.0·10^{-9}*	253	4.50	2.70 ÷ 6.88	*< 10^{-10}*
Pb	85	4.46 ± 2.94	3.44	1.36	2.38 ÷ 5.51	51	8.70	4.70 ÷ 43.00	*<10^{-10}*	336	21.00	11.95 ÷ 38.90	*< 10^{-10}*
S	65	1534 ± 237	1540	140	1371 ÷ 1650	11	1260	900 ÷ 1422	*0.002*	49	3170	2660 ÷ 4340	*< 10^{-10}*
Sb	63	0.093 ± 0.052	0.083	0.031	0.054 ÷ 0.118					53	0.220	0.170 ÷ 0.480	*< 10^{-10}*
Se	59	0.276 ± 0.095	0.270	0.051	0.220 ÷ 0.300					17	0.220	0.150 ÷ 0.350	0.458
Sn	62	0.335 ± 0.166	0.300	0.098	0.210 ÷ 0.410					38	0.495	0.320 ÷ 0.720	*1.4·10^{-6}*
Ti	64	11.1 ± 4.7	10.6	3.2	7.5 ÷ 13.8	23	98.2	47.3 ÷ 161.2	*<10^{-10}*	88	51.2	21.9 ÷ 96.5	*< 10^{-10}*
U	63	0.022 ± 0.013	0.020	0.008	0.011 ÷ 0.028	23	0.102	0.082 ÷ 0.185	*<10^{-10}*				
V	73	2.12 ± 0.47	1.96	0.13	1.90 ÷ 2.20					181	3.78	2.9 ÷ 5.7	*< 10^{-10}*
Zn	92	41.4 ± 17.4	39.8	13.3	27.0 ÷ 53.5	57	65.0	31.4 ÷ 118.0	*1.3·10^{-5}*	336	81.1	49.3 ÷ 129.9	*< 10^{-10}*

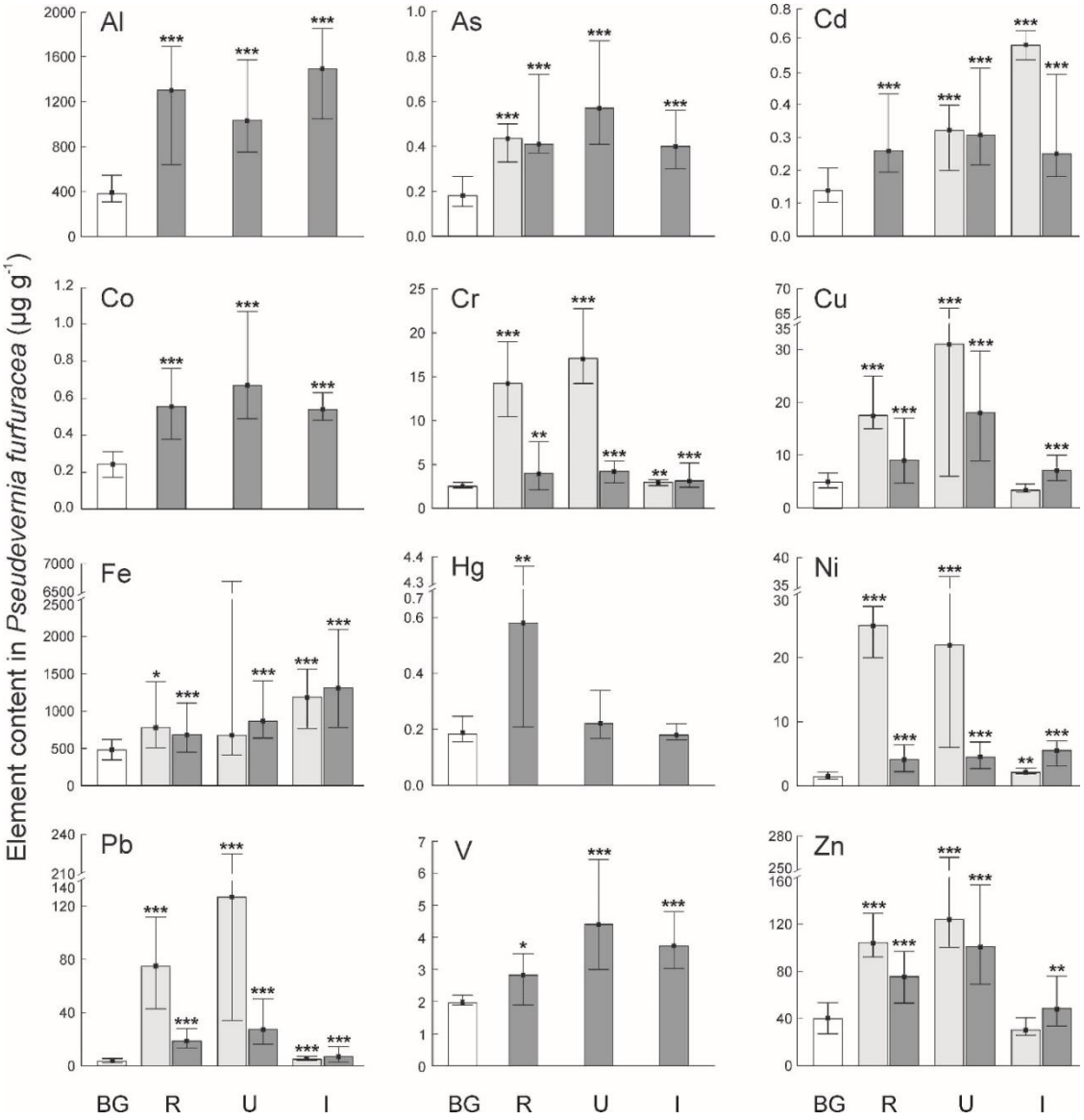


Figure 2. Comparative analysis of *Pseudevernia furfuracea* element content (µg g^{-1}) in background vs impacted conditions. Data refer to median and quartiles of element content distribution in the datasets BG ('background'; white bars), P ('passive'; light grey), and T ('transplants'; dark grey), the two latter separately calculated according to the land use at the collection/exposure sites: (BG: background areas; R: rural areas; U: urban areas; I: industrial areas). Data from the dataset P are not shown for Co, Cd, Hg, and V, due to limited sample size. Asterisks above bars indicate significant differences with respect to background conditions (Mann-Whitney U test, *: $0.01 < p < 0.05$; **: $0.001 < p < 0.01$; ***: $p < 0.001$).

3.3 Context-dependency of background element content at national level

The cluster analysis of element content data from the field sampling produced three main site clusters (Fig. 3A-B; Supplementary Fig. S2), well separated for geographical location, climatic conditions and lithological substrates. Cluster I included 22 sites generally characterized by metamorphic substrates in western Alps (except for sites 35 in eastern Alps, and 23 with sedimentary substrate); cluster II included 20 sites characterized by sedimentary carbonate substrates in eastern Alps and northern Apennines (with the exceptions of sites 4 in western Alps, and 27 with metamorphic substrate); cluster III included 20 sites characterized by different lithological substrates in the Apennines (with the exception of sites 7, 10 and 22, located in western Alps).

A clear pattern of lichen element composition along environmental gradients emerged from the PCA (Fig. 3C). In particular, elements of group 1 (Ag, Au, Cs, Rb, Bi, Sb, Sn, Cu, Mo, Zn) were consistently placed at high scores on the second PC axis, inversely related to temperature, and positively and negatively associated to the occurrence of metamorphic and carbonate substrates at the sampling sites, respectively. Differently, elements of groups 3 (Al, Ce, La, Y, Fe, Li, Ti, Th, Nb, U, Ca, Sr, Na, Hf, Zr) and 4 (As, Ge, Sc, V, Cd, Pb, S, Se, Hg) were positively associated to the first PC axis, consistent to prevalence of agricultural areas and low forest cover, high temperatures, and low precipitations. Elements of group 2 (Ba, Mn, Pd, Co, Cr, Ni, Mg, K, P) were not clearly associated to the first two PC axes, but inversely related to the abundance of carbonate substrates at the sampling sites, which mainly contributed to the third PC axis (8% of the total variance). In addition, K and P were negatively correlated with the fourth PC axis (6% of the total variance), hence positively to agricultural land cover and temperatures, and negatively to precipitations (data not shown). Interestingly, such environmental patterns of lichen element composition were generally corresponding to geographical gradients, as elements of group 1 showed also positive correlation with latitude and negative with longitude, whereas elements of groups 3 and 4 showed the opposite geographical pattern. Such geographical correspondence is better noticeable in the plot of collection sites in the ordination space (Fig. 3B).

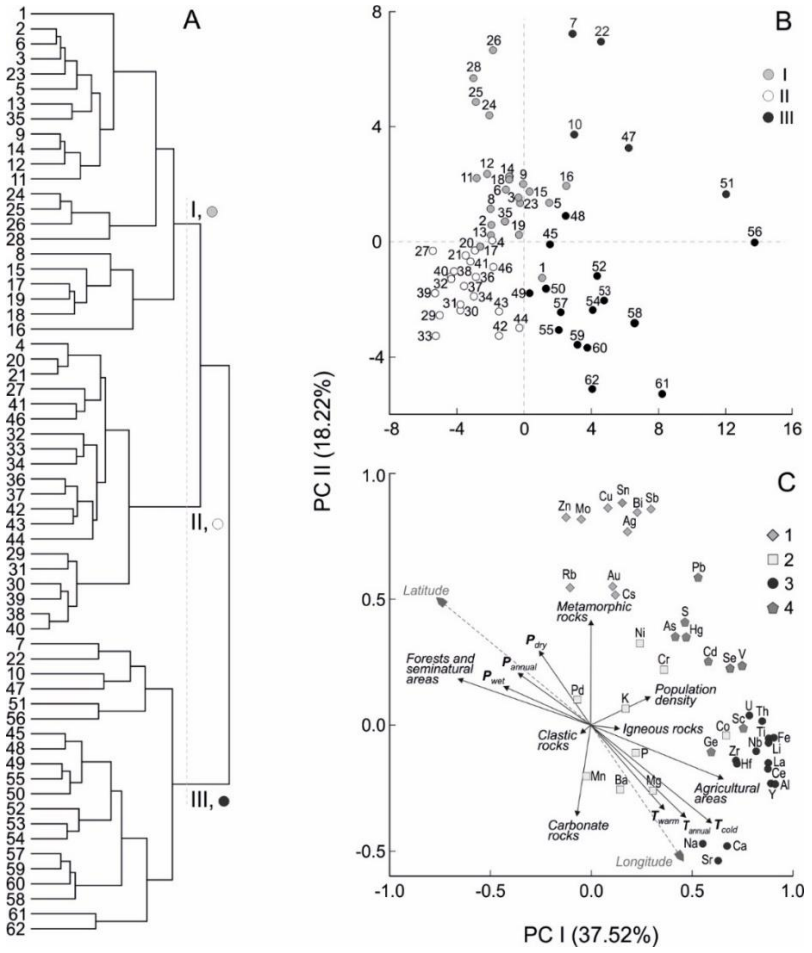


Figure 3. Results of multivariate analysis of element content in *Pseudevernia furfuracea* samples collected at 62 remote field sites in Italy. (A) Dendrogram of field sites from cluster analysis (CA), with three main clusters (I: ●; II: ○; III: ●); (B) factorial scores of field sites, symbolized according to CA results; (C) PCA plot showing loading vectors of the elements symbolized according to the CA of Fig. 5 and their relationships with environmental descriptors characterizing the field sites, plotted as supplementary variables following Legendre and Legendre (1998).

Consistent with the PCA results, clusters of sites at different geographic location (Supplementary Fig. S2) showed significant differences of standardized content of the three groups of elements (Fig. 4). In detail, sites of cluster I showed the highest mean content of elements of group 1, and intermediate values for the other element groups. Sites of cluster II showed the lowest content of all the element groups, while sites of cluster III showed the highest content of all the groups of elements, with the exception of group 1. Such pattern was consistent with environmental differences between clusters (Supplementary Table S4).

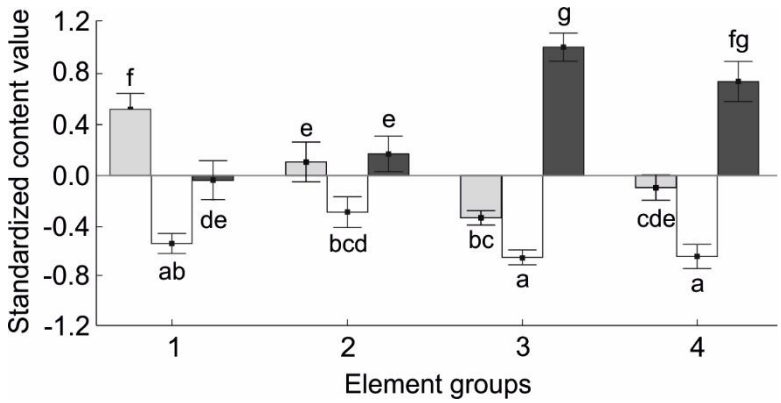


Figure 4. Content of element groups in the site clusters I-III of Fig. 3: data are separately standardized for each element and showed as mean and 95% confidence interval for 4 different element groups, as resulting from CA (Fig. 5). Letters above bars indicate significant pair-wise differences (Tukey's HSD test for unequal sample size, $p < 0.05$).

When removing from the clusters the few sites inconsistent for lithological substrate and/or location (i.e. sites 35, 23 from cluster I; sites 4, 27 from cluster II; sites 7, 10, 22 from cluster III), three sets of lichen samples were obtained, fully consistent for environmental conditions, collected respectively from: (1) western Alps, over metamorphic siliceous substrates, (2) eastern Alps and northern Apennines, over sedimentary rocks, and (3) central and southern Apennines, on different substrate types. Descriptive statistics for these sets of samples are proposed as BEC estimates at sub-national scale (Table 3). Notably, these showed significant between-group differences for 40 elements out of 43 (Table 3), with the exception of Ba, K and Pd. For comparison, comprehensive estimates at national scale are also provided (Supplementary Table S5).

Table 3. Background element content (μg g^{-1}) in the epiphytic lichen *Pseudevernia furfuracea* in Italy. These include sites with similar lichen element content according to CA results (Fig. 3A). Data refer to descriptive statistics (mean ± standard deviation, median, MAD, 98th percentile) for 43 elements, as suggested by Reimann et al. (2005). Between-groups differences (Kruskal-Wallis ANOVA; K-W, and significant *p*-values in italic) are reported for each element. Different letters indicate significantly different groups within each row (Dunn's post hoc test at $p < 0.05$).

	Siliceous metamorphic western Alps (n=20)				**Sedimentary eastern Alps and northern Apennines (n=18)**				**Central and southern Apennines (n=17)**				**K-W**
	Mean ± SD	**Median**	**MAD**	**98th %ile**	**Mean ± SD**	**Median**	**MAD**	**98th %ile**	**Mean ± SD**	**Median**	**MAD**	**98th %ile**	***p*-value**
Ag	0.025 ± 0.008	0.023 ^{b}	0.003	0.052	0.016 ± 0.007	0.014 ^{a}	0.002	0.040	0.020 ± 0.006	0.020 ^{ab}	0.003	0.003	*0.0001*
Al	359 ± 71	366 ^{a}	58	500	310 ± 78	300 ^{a}	48	500	758 ± 214	640 ^{b}	120	1200	*< 10^{-4}*
As	0.221 ± 0.081	0.207 ^{b}	0.042	0.408	0.143 ± 0.072	0.113 ^{a}	0.023	0.320	0.220 ± 0.091	0.220 ^{b}	0.060	0.450	*0.0015*
Au	0.180 ± 0.101	0.164 ^{b}	0.053	0.473	0.070 ± 0.062	0.055 ^{a}	0.028	0.260	0.130 ± 0.088	0.100 ^{ab}	0.048	0.320	*0.0004*
Ba	11.8 ± 4.6	11.6 ^{a}	1.2	26.9	12.7 ± 6.8	10.8 ^{a}	4.1	30.4	13.3 ± 7.7	9.3 ^{a}	4.3	29.1	0.8592
Bi	0.054 ± 0.017	0.051 ^{b}	0.011	0.099	0.032 ± 0.010	0.030 ^{a}	0.008	0.060	0.040 ± 0.018	0.040 ^{ab}	0.012	0.080	*0.0005*
Ca	4840 ± 962	4798 ^{a}	400	6900	6071 ± 2864	5909 ^{a}	1659	12860	11901 ± 4213	10487 ^{b}	1527	20780	*< 10^{-4}*
Cd	0.134 ± 0.031	0.137 ^{a}	0.020	0.200	0.114 ± 0.033	0.099 ^{a}	0.025	0.180	0.190 ± 0.053	0.170 ^{b}	0.020	0.310	*< 10^{-4}*

Table 3 (continued)

	Siliceous metamorphic western Alps (n=20)				Sedimentary eastern Alps and northern Apennines (n=18)				Central and southern Apennines (n=17)				K-W
	Mean ± SD	Median	MAD	98th %ile	Mean ± SD	Median	MAD	98th %ile	Mean ± SD	Median	MAD	98th %ile	*p*-value
Ce	0.79 ± 0.19	0.80 ^{a}	0.13	1.17	0.67 ± 0.22	0.63 ^{a}	0.08	1.39	1.98 ± 0.66	1.82 ^{b}	0.37	3.81	*< 10^{-4}*
Co	0.288 ± 0.131	0.261 ^{b}	0.058	0.750	0.166 ± 0.041	0.159 ^{a}	0.018	0.280	0.340 ± 0.090	0.310 ^{b}	0.072	0.510	*< 10^{-4}*
Cr	3.41 ± 1.59	2.85 ^{b}	0.35	9.54	2.48 ± 0.18	2.46 ^{a}	0.11	2.82	3.12 ± 0.46	3.00 ^{b}	0.18	4.45	*< 10^{-4}*
Cs	0.152 ± 0.108	0.098 ^{b}	0.032	0.383	0.073 ± 0.040	0.062 ^{a}	0.011	0.200	0.120 ± 0.065	0.100 ^{b}	0.024	0.300	*0.0007*
Cu	7.05 ± 2.26	6.79 ^{b}	1.37	12.51	4.23 ± 1.01	3.88 ^{a}	0.21	7.32	4.69 ± 1.51	4.24 ^{a}	0.90	8.70	*< 10^{-4}*
Fe	468 ± 112	481 ^{b}	78	726	352 ± 90	348 ^{a}	45	600	830 ± 246	762 ^{c}	118	1385	*< 10^{-4}*
Ge	0.012 ± 0.004	0.012 ^{a}	0.002	0.023	0.012 ± 0.002	0.011 ^{a}	0.001	0.020	0.020 ± 0.004	0.020 ^{b}	0.002	0.030	*0.0002*
Hf	0.053 ± 0.024	0.049 ^{b}	0.012	0.121	0.036 ± 0.015	0.032 ^{a}	0.006	0.090	0.080 ± 0.025	0.070 ^{c}	0.017	0.130	*< 10^{-4}*
Hg	0.208 ± 0.040	0.200 ^{b}	0.026	0.301	0.171 ± 0.059	0.164 ^{a}	0.020	0.350	0.240 ± 0.036	0.250 ^{b}	0.015	0.310	*< 10^{-4}*
K	3309 ± 427	3360 ^{a}	343	4114	3235 ± 500	3255 ^{a}	493	4180	3676 ± 645	3550 ^{a}	337	4720	0.0717
La	0.354 ± 0.098	0.340 ^{a}	0.062	0.604	0.279 ± 0.103	0.261 ^{a}	0.041	0.620	0.900 ± 0.311	0.840 ^{b}	0.126	1.840	*< 10^{-4}*
Li	0.293 ± 0.071	0.291 ^{a}	0.052	0.450	0.215 ± 0.063	0.212 ^{a}	0.027	0.370	0.540 ± 0.154	0.500 ^{b}	0.066	0.950	*< 10^{-4}*
Mg	778 ± 274	661 ^{a}	76	1520	753 ± 138	721 ^{ab}	81	1054	878 ± 152	830 ^{bc}	88	1248	*0.0153*
Mn	64.2 ± 18.3	62.8 ^{bc}	13.7	101.5	58.6 ± 30.2	46.7 ^{ab}	14.6	132.6	50.7 ± 45.9	35.1 ^{a}	11.9	175.7	*0.0083*
Mo	0.380 ± 0.129	0.358 ^{b}	0.064	0.790	0.165 ± 0.049	0.165 ^{a}	0.030	0.290	0.150 ± 0.062	0.120 ^{a}	0.024	0.270	*< 10^{-4}*
Na	34.9 ± 24.3	30.0 ^{a}	5.8	134.3	65.9 ± 50.9	48.1 ^{a}	18.8	191.4	179.9 ± 38.5	174.0 ^{b}	16.0	273.7	*< 10^{-4}*
Nb	0.033 ± 0.007	0.034 ^{b}	0.004	0.046	0.026 ± 0.019	0.022 ^{a}	0.005	0.100	0.070 ± 0.022	0.070 ^{c}	0.010	0.130	*< 10^{-4}*
Ni	2.06 ± 1.06	1.52 ^{b}	0.39	3.85	0.85 ± 0.31	0.76 ^{a}	0.15	1.74	1.31 ± 0.33	1.26 ^{b}	0.20	2.10	*< 10^{-4}*
P	550 ± 145	512 ^{ab}	52	1060	518 ± 161	467 ^{a}	85	901	668 ± 191	622 ^{b}	67	1151	*0.0101*
Pb	3.15 ± 1.902	3.36 ^{b}	0.64	5.51	2.19 ± 0.57	2.12 ^{a}	0.38	3.64	3.66 ± 1.65	3.05 ^{b}	0.68	8.42	*0.0002*
Pd	0.0033 ± 0.0016	0.0027 ^{a}	0.0006	0.0065	0.0026 ± 0.0009	0.0023 ^{a}	0.0003	0.0053	0.0024 ± 0.0007	0.0022 ^{a}	0.0003	0.0042	0.1070
Rb	15.6 ± 7.6	14.0 ^{b}	3.7	33.8	7.5 ± 4.2	6.1 ^{a}	1.7	17.8	10.0 ± 5.8	7.1 ^{a}	3.3	20.9	*0.0018*
S	1508 ± 15	1535 ^{ab}	90	1760	1370 ± 189	1340 ^{a}	117	1680	1640 ± 210	1600 ^{b}	100	2260	0.0006
Sb	0.119 ± 0.039	0.115 ^{b}	0.025	0.224	0.055 ± 0.016	0.054 ^{a}	0.009	0.090	0.080 ± 0.046	0.070 ^{a}	0.026	0.190	*< 10^{-4}*
Sc	0.342 ± 0.032	0.337 ^{a}	0.017	0.420	0.324 ± 0.041	0.316 ^{a}	0.016	0.420	0.410 ± 0.050	0.400 ^{b}	0.040	0.500	*< 10^{-4}*
Se	0.259 ± 0.044	0.268 ^{b}	0.033	0.342	0.209 ± 0.053	0.218 ^{a}	0.025	0.340	0.410 ± 0.139	0.420 ^{c}	0.130	0.610	*< 10^{-4}*
Sn	0.425 ± 0.142	0.408 ^{b}	0.086	0.821	0.246 ± 0.055	0.257 ^{a}	0.042	0.370	0.270 ± 0.164	0.230 ^{a}	0.079	0.660	*< 10^{-4}*
Sr	9.7 ± 2.1	9.9 ^{a}	1.3	13.3	13.9 ± 5.9	12.7 ^{a}	2.6	28.1	26.4 ± 9.7	24.7 ^{b}	4.6	49.5	*< 10^{-4}*
Th	0.085 ± 0.027	0.081 ^{b}	0.022	0.148	0.056 ± 0.015	0.055 ^{a}	0.007	0.100	0.170 ± 0.068	0.160 ^{c}	0.020	0.340	*< 10^{-4}*
Ti	10.4 ± 2.4	10.1 ^{b}	1.6	15.0	7.0 ± 1.7	6.8 ^{a}	0.9	10.7	16.8 ± 4.1	17.2 ^{c}	2.8	26.6	*< 10^{-4}*
U	0.020 ± 0.009	0.020 ^{b}	0.004	0.050	0.011 ± 0.003	0.010 ^{a}	0.001	0.020	0.030 ± 0.012	0.030 ^{c}	0.007	0.060	*< 10^{-4}*
V	2.23 ± 0.51	2.04 ^{b}	0.10	3.97	1.94 ± 0.12	1.90 ^{a}	0.00	2.40	2.60 ± 0.65	2.50 ^{b}	0.50	3.97	*< 10^{-4}*
Y	0.470 ± 0.153	0.448 ^{a}	0.121	0.875	0.363 ± 0.109	0.349 ^{a}	0.031	0.720	0.930 ± 0.309	0.900 ^{b}	0.156	1.850	*< 10^{-4}*
Zn	48.5 ± 14.9	43.9 ^{b}	7.8	83.6	30.4 ± 9.4	27.3 ^{a}	9.0	45.3	24.5 ± 6.5	24.4 ^{a}	5.4	36.9	*< 10^{-4}*
Zr	2.29 ± 0.90	2.15 ^{b}	0.55	4.18	1.53 ± 0.51	1.35 ^{a}	0.19	2.94	3.13 ± 1.00	2.74 ^{b}	0.43	5.31	*< 10^{-4}*

3.4 Relationships between environmental predictors and background element content

All environmental variables were significantly associated with lichen element content, being predictive for at least three chemical elements (Fig. 5). A consistent pattern of correlation was found between single environmental variables and elements in the same groups. Indeed, population density was consistently positively associated to lichen element content, particularly with element groups 3 and 4 (Fig. 5). Land use in the surroundings of the sampling sites differently affected the lichen content of different groups of elements, with values increasing with increasing agricultural land cover, and decreasing with increasing forest cover, for all elements of group 3, and most elements of groups 4 and 2, whereas elements of groups 1 were inversely or not affected (Fig. 5).

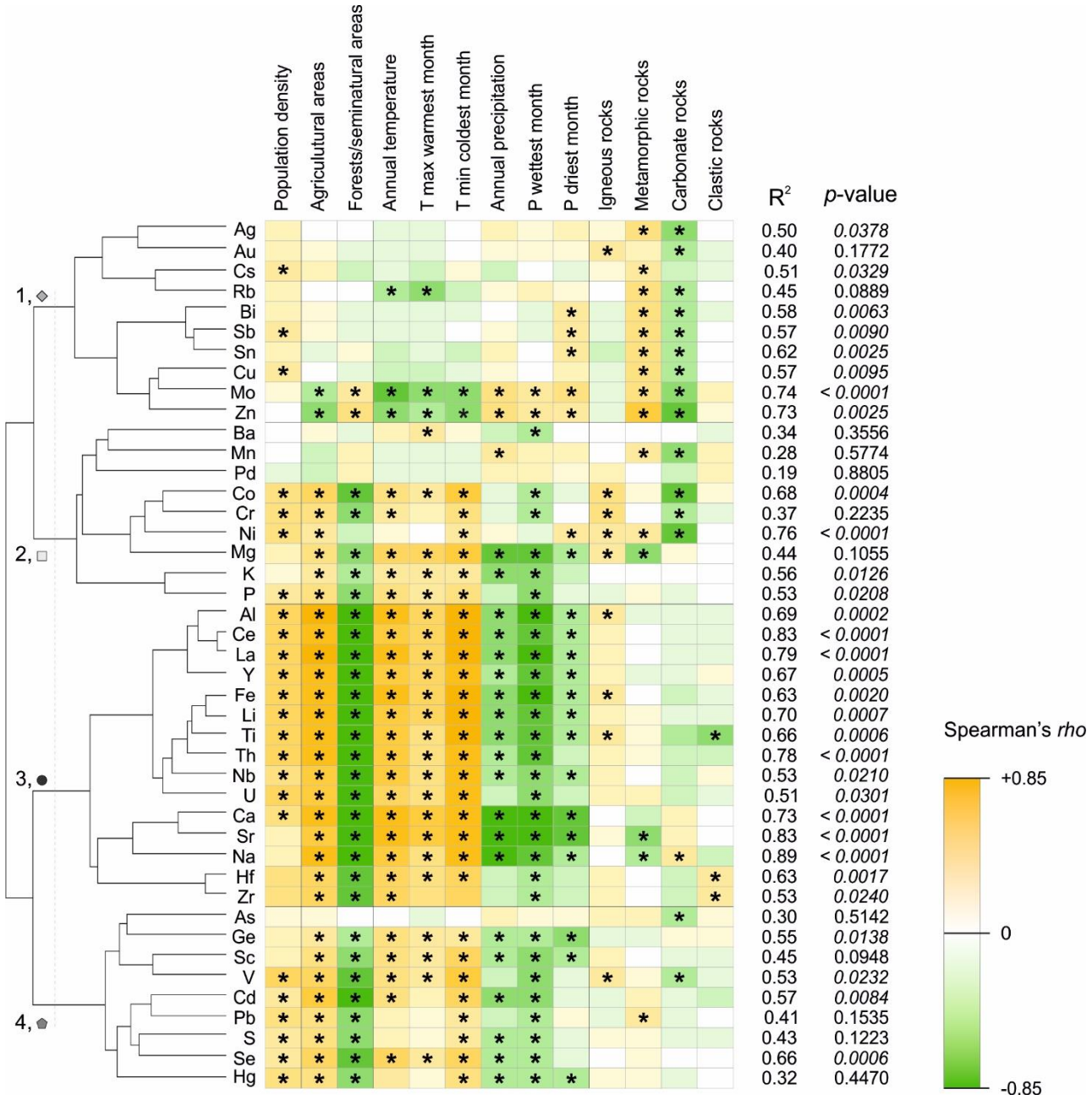


Figure 5. Heatmap showing the association between *Pseudevernia furfuracea* element content and environmental descriptors at the field sites. Dendrogram of the elements based on content values in the lichen at 62 field sites is also shown. Asterisks indicate significant rank correlation values (Spearman's *rho*, $p < 0.05$), either positive (orange) or negative (green). For each element, the predictivity (multiple-R^2 and associated *p*-values, significant values in italic) of a Principal Component Regression model based on the set of environmental descriptors is also shown.

Lichen content of elements of groups 2, 3, and 4 was also significantly affected by climatic variables, with a pattern of positive and negative correlation for temperatures and precipitations, respectively. These were consistent for all elements of groups 3 and 4, except for As, with the formers more frequently associated to mean annual temperature, the latter to minimum temperature of the coldest month, and both groups to total precipitations of the wettest month (Fig. 5). A very similar pattern to that of group 4 was found for most elements of group 2 (Ba, Co, Cr, Mg, K, P and, limited to temperatures, Ni), while lichen content of those of group 1 was generally not (Ag, Au, Cs, Bi, Sb, Sn, Cu) or negatively (Rb, Mo, Zn) correlated to mean and maximum temperatures, and positively associated to precipitations (limited to Bi, Sb, Sn, Mo, Zn).

Lichen content of elements of group 1 were positively and negatively associated to metamorphic and carbonate rocks, respectively. A similar pattern of correlation was found for some elements of group 2 (Mn, Ni and, limited to carbonate substrate, Co and Cr), while others were positively associated to metamorphic rocks (Mg) or negatively to igneous rocks (Co, Cr, Ni, Mg). Differently, lichen content of elements of groups 3 and 4 was barely affected by lithology, with few significant correlation scores of opposite signs scattered within the groups for metamorphic (positive for Pb and negative for Sr and Na), carbonate (positive for Na and negative for As and V) and clastic (positive for Hf and Zr and negative for Ti) rocks. Finally, igneous rocks were positively correlated with typical terrigenous elements (Al, Fe, Ti) and V.

Considering multivariate environmental relationships as assessed by PCR modelling, statistically significant outcomes were found for 31 out of the 43 tested elements (Fig. 5, detailed results in Supplementary Table S6). Exceptions, unsatisfactorily related to environmental predictors, were Au and Rb (group 1), Ba, Mn, Pd, Cr and Mg (group 2) and As, Sc, Pb, S, and Hg (group 4). Interestingly, statistically significant PCR models were found for all elements of group 3, mostly including elements of lithogenic origin and rare earth ones. In 10 cases (i.e. Al, Ca, Hf, La, Li, Na, Nb, Th, Ti and Zr) the PCR models were also significantly predictive of the validation datasets (Supplementary Fig. S3-S6). Differently, PCR models for elements of other groups, although significantly predictive of the fitting datasets, did not provide satisfactory performance when applied to the validation datasets (Supplementary Fig. S3-S6) in terms of predicted vs observed comparisons.

4. Discussion

4.1 Estimate of background element content at large scale: methodological insights

Our BG dataset provides for the first time a broad overview of the BEC ranges at supranational scale for a single, highly performing lichen biomonitor which is widely used in active and passive biomonitoring surveys. The papers selected for the set-up of this dataset, obviously heterogeneous in scopes and objectives, showed a noteworthy methodological heterogeneity, with widely different procedures for sample pre-processing, digestion, analytical techniques, and QC assessment (Table 1). This could heavily affect the comparability of analytical results (e.g., Baffi et al. 2002; Bettinelli et al. 2002), which was addressed by a clean-up procedure, as described in Supplementary Material, Sect. S1.4. Besides, prior to calculate the BEC statistical descriptors, we removed superior extreme outliers (OLs) from the data distribution of each element, as possibly produced by instrumental bias, mistaken reports, or unexpected anthropogenic contributions at the sampling sites, thus producing unbiased element data distributions, and therefore more reliable BEC ranges. In the literature on robust statistics, many approaches for outlier detection have been proposed (e.g., Barnett and Lewis 1994; Dutter et al. 2003). Here we used Tukey's method, which makes no distributional assumptions, thus being applicable to skewed or non-bell-shaped data distributions (Hoaglin et al. 1986; Reimann et al. 2005).

4.2 Comparison of background and enriched element content at large scale

The definition of BEC values is intimately connected to the practical differentiation between naturally (bio-)geogenic and anthropogenically-influenced element concentrations (Matschullat et al. 2000). Although in principle it is almost impossible to quantify a true BEC value beyond doubt, it seems possible to derive a plausible, realistic approximation by comparing (and eventually merging) different data-sets. Expectedly, our comparative analysis of element content data confirmed that BG data were substantially unaffected by anthropogenic contributions, as they were significantly lower for most elements in comparison to P and T data. The only exceptions were K, Mn, S, Se and, partially, Hg. Potassium and Mn typically decreases in lichens exposed to airborne pollutants due to membrane leakage (e.g. Garty et al. 1998; Häffner et al. 2001), contextually with vitality loss (Bari et al. 2001), as a result of washing effects by rainfalls (Gallo et al. 2017). Concerning S and Se, their content can be highly heterogeneous, being influenced by different natural and anthropical sources, transport phenomena, and by the physiological state of the thalli (Låg and Steinnes 1974; Vingiani et al. 2004; Brenot et al. 2007; Wen and Carignan 2007). Finally, high Hg levels are usually related to wet depositions, altitude and geochemical anomalies (Bargagli 2016; Carasci and Cataldo 2016; Zechmeister 1995), possibly affecting data distribution in the T dataset.

4.3 Relationships between environmental descriptors and background element content

At confirmation that *Pseudevernia furfuracea* is a biomonitor sensitive to minimal environmental differences, we found a pattern of association between the (generally low) BECs and the site conditions in remote areas. In particular, land use, climate and lithology were satisfactorily predictive, confirming the findings of Incerti et al. (2017). The effects of these factors on lichen bioaccumulation are widely acknowledged in the literature (e.g. Garty 2001; Nimis 2001; Sorbo et al. 2008; Agnan et al. 2014), but they were neglected in the build-up of BEC values and interpretative scales (Nimis and Bargagli 1999).

In our national dataset of BEC values, elemental content generally increases with increasing population density, temperatures, cover of agricultural areas and metamorphic substrate (hence, moving southwards), whereas it decreases with increasing precipitation, forests and carbonate substrates cover (hence, moving northwards, and eastwards within the Alps). Sites of central-southern Italy showed consistently higher content of lithogenic (Al, Ca, Fe, Li, Th, and Ti) and rare earth elements (REE: Ce, La, Sc, Y), as related to higher levels of anthropization, agricultural landcover, and soil susceptibility to erosion (Jones et al. 2012; Capozzi et al. 2016), as well as lower precipitations. REE and lithogenic elements are often considered tracers of geochemical transport processes (Aubert et al. 2001; Laveuf and Cornu 2009). Therefore, central and southern sites, often located along slopes prone to upward blowing winds from relatively arid rural lowlands, may be affected by important depositions of windblown dust. Moreover, bedrock weathering and soil erosion, in areas characterized by crystalline and marine clay rich sediments outcrops as well as by a high incidence of rural activities, may be major sources of REE. Indeed, some REE such as Ce, La and Y are also known for being included in phosphate fertilizers and insecto-fungicides (Sadeghi et al. 2013; Carpenter et al. 2015; Di Palma et al. 2017), which could explain higher background levels

in samples from sites subjected to influence by agricultural areas. This is further supported by the higher background levels of P in central and southern sites, compared to northern ones. These evidences suggest that local background of central and southern Italy is likely affected by medium-long-range depositions.

When considering the northern Italian sites, we observed a general pattern of higher vs lower background levels at western vs eastern sites, respectively, for 25 out of 43 elements, while no element showed the opposite trend. Such pattern, clearly reflects the main lithological traits of local substrates, with western sites laying over siliceous metamorphic rocks, and eastern sites over sedimentary carbonates, respectively. Although the traceability of rare elements may be questionable, this pattern is particularly evident for Ag, Au, Bi, Cu, Mo, Rb, Sb, Sn and Zn, which occur into different metamorphic substrate types, as in the cases of Cu, Rb and Sb (Aubert and Pinta 1980; Bargagli et al. 1999; Salminen et al. 2005; Kuleshov 2016), and Mo into sulphides pyrite, galena and sphalerite (Salminen et al. 2005). Consistently, previous observations on element content in mosses showed that differences at regional scale are associated to the main geochemical traits (Bargagli 1995).

Our PCR modelling of element background levels at national scale showed reliable results for most lithogenic elements and REE (Al, Ca, Hf, La, Li, Nb, Sb, Th, Ti, Zr) and for Na, with models for the fitting dataset being also predictive of the validation datasets. In the case of Na, the scatterplot of observed vs predicted values clearly separated Alpine from Apennine sites, with the latter consistently showing higher Na levels. Besides environmental predictors included in the PCR model, an effect of long-range transport of marine aerosol by prevailing south-westerly winds from the Tyrrhenian sea (Zecchetto and Cappa 2001) should not be excluded. On the other hand, the common pattern observed for lithogenic and REE elements reinforce our hypothesis on the common source and transport phenomena for such elements. Finally, PCR models for As, Au, Bi, Co, Cs, Mg, Pd, and V showed the worst performance. Therefore, further investigation is recommended to explore the relationships between the BEC of these elements and other environmental factors.

4.4 Background element content as an interpretative tool in biomonitoring application

The knowledge of chemical background is an essential pre-requisite for the assessment of pollution levels and possible biological effects (Bargagli 1998). Our results on BEC in *P. furfuracea* at supra-regional scale provide a main advance in this direction, highlighting significant differences among three Italian macro-regions for most elements due to a remarkable context-dependency of BEC values. This agrees well with the observation of Reimann et al. (2005) that in naturally geochemically complex or large survey areas there may be multiple discrete background populations, which need to be properly recognized. As such, the BEC values reported in Table 3 should be regarded as reference datasets for biomonitoring application with *P. furfuracea* in Italy as well as in areas with the same combinations of environmental conditions at supra-national level. In particular, the use of our data is recommended at the interpretation stage of biomonitoring results. In the case of passive biomonitoring application, the comparison of enriched samples with our geographically-consistent background estimate is suggested in place of commonly used multi-species interpretative scales (Nimis and Bargagli 1999), possibly integrated by the use of graphical inspection for dataset filtering, like those applied by Reimann et al. (2005). In the case of

transplants, element content in pre-exposure samples should be carefully compared to our background estimates for the selection of a suitable sampling area, in order to avoid the use of samples with high levels of specific elements which might interfere with those to be measured in the transplant experiment (Frati et al. 2005). On the other hand, further collection of element content data in native lichen samples, based on highly standardized methodological protocols, could help to build up reliable interpretative tools, such as single-species scales (Nimis et al.2001; Tretiach and Baruffo 2001a), which were not presented in this contribution due to sample size constraints. Indeed, it could be possible to compare the element content data in native lichen samples, with the distribution of total element concentration in top-soils of Europe (Salminen et al. 2005; De Vos et al. 2006), while the collection of new data at very local scale coupled with environmental datasets at high spatial resolution should allow to provide accurate BEC estimates for more restricted and environmentally homogenous contexts.

5. Conclusions

Our BG dataset provides for the first time a broad overview of the BEC ranges at supranational scale for a single, highly performing lichen biomonitor, *P. furfuracea*, which is widely used in active and passive biomonitoring surveys throughout Europe and extra-European countries. The extensive review of active and passive bioaccumulation studies revealed a huge methodological variability. By compiling and comparatively analysing methodologically homogeneous datasets, however, we could provide a first, comprehensive overview of BEC ranges for 25 chemical elements at supra-national level. Limited to original field data from Italy, we explored the background levels for 43 elements in relation to environmental conditions at the sampling sites, finding high predictivity of anthropization, land use, climate and lithological variables for most elements. We also identified three homogeneous and geographically separated contexts (western Alps, eastern Alps plus northern Apennines, central and southern Apennines), for which specific BEC values are now available as reference datasets for biomonitoring applications.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS S1

S1.1 The target species

Pseudevernia furfuracea is a fruticose, meso-xerophilous and photophilous species, occurring in two morphologically undistinguishable varieties (var. *furfuracea* and var. *ceratea*) mainly on acidic, non-eutrophicated bark. The species has a wide cool-temperate/boreal distribution (Rikkinen 1997; Smith et al. 2009). The thalli are richly branched and the upper surfaces are often densely covered by finger-shaped outgrowths (isidia), which considerably increase the exchange surface per area and mass unit (Tretiach et al. 2005) and the particle entrapment (Bargagli and Mikhailova 2002; Riga-Karandinos and Karandinos 1998). The large thallus size and the easy identification in the field ensure faster and easier sampling and preparation, compared to other lichens. In addition, the availability of very flourishing, abundant populations in temperate/boreal forests definitely limit the risk of their impoverishment due to dedicated sampling campaigns. Not surprisingly, therefore, *P. furfuracea* has been targeted in several methodological studies, aimed at improving data quality and standardizing protocols for lichen transplants (e.g., Adamo et al. 2007; Adamo et al. 2008; Incerti et al. 2017), and is frequently used in biomonitoring surveys of trace elements. For all these features, this species is among the best candidates for an application-driven determination of BEC values.

S1.2 Processing data from previous studies

Studies resulting from the literature search (see Sect. 2.1 in main text) were individually reviewed and excluded from further analysis if the data were: (*i*) referring to other taxa; (*ii*) derived from pooled lichen species; (*iii*) expressed in relative terms (e.g. EC ratio); (*iv*) limited to radioisotopes and radionuclides.

For the selected studies, all element content data were considered; values reported in the papers as being below the limit of instrumental detection (LOD) were either rounded to the highest value below the LOD at the highest number of significant digits (e.g. 0.019 for values below a LOD of 0.020) when the LOD was reported in the paper, or discarded when LOD was not reported. When element concentration was given as plotted data, estimated values were extracted using Web Plot Digitizer software (Rohatgi 2011). All data were expressed in $\mu g\ g^{-1}$. Ancillary information reported in the original papers was also recorded, including data metrics (e.g. raw values, mean, median, range for different replicates, samples and/or sites), location (country, geographic coordinates, altitude) and land use of collection and/or exposure sites, host tree species and, limited to active biomonitoring studies, lichen exposure devices (e.g. bags or other devices) and duration of the exposure. We also recorded relevant methodological information, concerning (*i*) pre-treatments of lichen material (e.g. washing, drying; see Adamo et al. 2008), (*ii*) protocol of element content determination (e.g. acid digestion procedure), (*iii*) analytical technique (Baffi et al. 2002; Yafa and Farmer 2006), and (*iv*) quality control (QC) procedures. In the case of acid digestion, acid mixtures were divided in two groups, according to the presence/absence of hydrofluoric acid (Niazi et al.

1993; Cook et al. 1997; Perez-Santana et al. 2007): (*i*) 'total' digestions, with HF, and (*ii*) 'partial' digestions, without HF (often referred as 'aqua regia' or 'strong acid digestion'). About QC procedures, studies were partitioned into six groups, according to increasing details provided on the use of certified reference material (CRM) and assessment of element content percentage recovery (Quevauviller et al. 1996).

S1.3 GIS-based environmental characterization of the field sites

The 62 collection sites were characterized in terms of anthropization, land use, climate, and lithology, using thematic maps in a GIS environment. All GIS analyses were performed using QGIS 2.18.9 'Las Palmas' software.

Anthropization was indirectly estimated within three buffers of increasing radius (5, 10 and 25 km) centered on each collection site, considering: (a) population density (inhabitants per km^2) calculated from national census data (ISTAT, https://www.istat.it), as the sum of inhabitants of census units completely or partially included in the buffers, weighed by the relative census unit area included in the buffer, divided by the buffer area; (b) built-up area cover for residential, productive and scattered buildings, considering their percent contributions within the buffers as described above. Within the same buffers, the area covered by artificial surfaces, agricultural areas, and forest and semi-natural areas was calculated from Corine Land Cover map (CLC 2012, Bossard et al. 2000; http://land.copernicus.eu/), considering first level classes 1, 2 and 3, respectively.

Climatic data were downloaded from publicly available repositories (O'Donnel et al. 2012; www.worldclim.org). The following variables were considered, mapped at 30-arcseconds spatial resolution (≈ 1 km) and calculated over the period 1960-1990: annual temperature, maximum temperature of the warmest month, minimum temperature of the coldest month, annual precipitation, precipitation of the wettest month and precipitation of the driest month.

For the lithological characterization of the sampling sites, the geological map of Italy at 1:1˙000˙000 was used (ISPRA, sgi.isprambiente.it). The collection sites were characterized considering the percent cover of igneous, metamorphic, sedimentary carbonate and sedimentary clastic rocks within three buffers of increasing radius (0.5, 1 and 5 km, respectively) centered on each site.

Overall, a total of 39 environmental variables were considered (i.e. 3 buffer sizes × population density, 3 built-up area classes, 3 land use classes and 4 lithological classes, plus 6 bioclimatic variables). Limited to field data, a preliminary assessment of Spearman's rank correlation between lichen element content and the 39 environmental variables characterizing the collection sites, led to the selection for further analyses of 13 potential predictors, as most associated to lichen element content. These were spatially referred to either grid cells or buffers of different size, as follows: population density (25 km), percent area cover of agricultural areas, and forests and seminatural areas (25 km), 6 climatic variables (1 km), percent area cover of igneous, metamorphic, sedimentary carbonate and clastic rocks (0.5 km).

S1.4 Data cleaning in the BG dataset

In order to minimize possible bias in the BG dataset, we excluded all data derived from total acid digestion, as this treatment, compared to weaker acid mixtures (used for the majority of our records), is known to produce higher percent recovery for some elements (e.g. Al, Cr, Cu, Fe, Mn, Ti, V, and Zn in BCR 482; Baffi et al. 2002). Moreover, we removed superior extreme outliers (OLs) for each element, as possibly affected by instrumental biases, typing errors, oversights in reporting units of measure etc., as well as by unexpected anthropogenic contributions at the sampling sites. Extreme OLs were identified according to the Tukey's method (i.e. values higher than the 3rd quartile of the distribution plus 3 times the interquartile range), which makes no distributional assumptions, thus being applicable to skewed or non-bell-shaped data distributions (Hoaglin et al. 1986). Finally, the elements with low sample size ($n < 30$), or with at least 50% of field values below the LOD were excluded from further data analysis).

SUPPLEMENTARY TABLES S1-S6

Supplementary Table S1. List of the sampling sites of the epiphytic lichen *Pseudevernia furfuracea*, with location, environmental descriptors and collectors.

Site ID	Region (province)	Municipality	UTM (E)	UTM (N)	Altitude (m a.s.l.)	Anthropization	Land use		Climate		Lithological substrate	Collectors
						Population density (km^{-2})	Agricultural areas (%)	Forests/seminatural areas (%)	Annual temperature (°C)	Annual precipitation (mm)		
1	Piemonte (CN)	Sampeyre	355214	4936081	1400	36.6	13.2	85.7	6.5	1014	Metamorphic	Favero Longo S.
2	Piemonte (TO)	Perrero	352273	4972752	1985	83.8	16.8	80.8	3.4	1287	Metamorphic	Bidussi M., Capozzi F.
3	Piemonte (TO)	Perrero	352389	4972481	2100	83.8	17.0	80.6	2.6	1356	Metamorphic	Bidussi M., Capozzi F.
4	Piemonte (TO)	Perrero	352790	4973901	1780	86.1	17.5	80.1	4.1	1228	Metamorphic	Bidussi M., Capozzi F.
5	Piemonte (TO)	Perrero	352934	4975870	1300	95.3	17.7	79.4	7.2	980	Metamorphic	Bidussi M., Capozzi F.
6	Piemonte (TO)	Perrero	353312	4975156	1560	90.9	18.4	79.0	6.3	1049	Metamorphic	Bidussi M., Capozzi F.
7	Piemonte (TO)	Groscavallo	364072	5025600	1175	19.7	3.7	95.7	7.8	965	Metamorphic	Favero Longo S.
8	Val d'Aosta (AO)	Morgex	344106	5066088	1550	44.3	8.0	90.7	2.0	1586	Sedim. clastic	Favero Longo S.
9	Lombardia (SO)	Fusine	558229	5106887	1205	62.1	8.7	89.4	6.7	1004	Metamorphic	Capozzi F., Panepinto F.
10	Lombardia (SO)	Fusine	558929	5103665	1610	65.5	9.5	88.5	4.8	1013	Metamorphic	Capozzi F., Panepinto F.
11	Lombardia (SO)	Fusine	558949	5104594	1425	63.4	9.2	88.9	5.5	1002	Metamorphic	Capozzi F., Panepinto F.
12	Lombardia (SO)	Fusine	558689	5101601	2000	73.5	10.0	87.9	2.0	1126	Sedim. clastic	Capozzi F., Panepinto F.
13	Lombardia (SO)	Fusine	559195	5101932	1820	73.1	10.0	87.9	3.7	1044	Metamorphic	Capozzi F., Panepinto F.
14	Lombardia (BG)	Foppolo	559576	5099879	1950	75.3	10.5	87.3	3.6	1045	Sedim. clastic	Capozzi F., Panepinto F.
15	Lombardia (SO)	Lanzada	569126	5125647	1250	68.4	9.0	89.3	6.4	933	Sedim. clastic	Capozzi F., Panepinto F.
16	Lombardia (SO)	Lanzada	570601	5126912	1530	68.1	9.1	89.2	5.0	918	Igneous intrusive	Capozzi F., Panepinto F.
17	Lombardia (SO)	Lanzada	571172	5126457	1750	66.7	9.2	89.1	3.4	962	Igneous intrusive	Capozzi F., Panepinto F.
18	Lombardia (SO)	Lanzada	572129	5128037	2120	68.2	9.6	88.7	1.8	1065	Igneous intrusive	Capozzi F., Panepinto F.
19	Lombardia (SO)	Lanzada	572158	5128410	1990	68.5	9.7	88.7	1.8	1065	Igneous intrusive	Capozzi F., Panepinto F.
20	Trentino Alto Adige (TN)	Trento	657706	5100843	1170	184.5	19.9	74.5	7.0	748	Sedim. carbonate	Cristofolini F.
21	Trentino Alto Adige (TN)	Trento	658302	5099679	1635	183.9	19.7	74.8	4.3	737	Sedim. carbonate	Cristofolini F.
22	Trentino Alto Adige (TN)	Trento	658562	5099302	1780	182.6	19.6	75.0	4.3	737	Sedim. carbonate	Cristofolini F.
23	Trentino Alto Adige (TN)	Trento	658610	5099527	1735	182.4	19.6	74.9	4.3	737	Sedim. carbonate	Cristofolini F.
24	Trentino Alto Adige (BZ)	San Leonardo in Passiria	675604	5189316	1720	58.0	11.0	87.8	1.5	997	Metamorphic	Candotto Carniel F., Craighero T.
25	Trentino Alto Adige (BZ)	San Leonardo in Passiria	675848	5188959	1635	57.6	11.2	87.6	4.2	829	Metamorphic	Candotto Carniel F., Craighero T.
26	Trentino Alto Adige (BZ)	San Leonardo in Passiria	676278	5189427	1845	57.2	10.8	87.9	1.4	999	Metamorphic	Candotto Carniel F., Craighero T.
27	Trentino Alto Adige (BZ)	San Leonardo in Passiria	676607	5188632	1630	56.7	11.2	87.6	3.5	861	Metamorphic	Candotto Carniel F., Craighero T.
28	Trentino Alto Adige (BZ)	San Leonardo in Passiria	676711	5189071	1820	56.5	10.9	87.8	2.2	940	Metamorphic	Candotto Carniel F., Craighero T.

Supplementary Table S1 (continued)

Site ID	Region (province)	Municipality	UTM (E)	UTM (N)	Altitude (m a.s.l.)	Anthropization	Land use		Climate		Lithological substrate	Collectors
						Population density (km^{-2})	Agricultural areas (%)	Forests/seminatural areas (%)	Annual temperature (°C)	Annual precipitation (mm)		
29	Veneto (BL)	Rocca Pietore	721351	5144028	1965	30.0	6.8	91.9	2.3	992	Sedim. carbonate	Bidussi M., Capozzi F.
30	Veneto (BL)	Rocca Pietore	721655	5144322	1835	30.0	6.8	91.8	3.1	926	Sedim. carbonate	Bidussi M., Capozzi F.
31	Veneto (BL)	Rocca Pietore	721851	5144747	1660	30.0	6.9	91.8	3.9	874	Sedim. carbonate	Bidussi M., Capozzi F.
32	Veneto (BL)	Rocca Pietore	722894	5145358	1470	29.4	6.7	92.0	3.7	888	Sedim. carbonate	Bidussi M., Capozzi F.
33	Veneto (BL)	Rocca Pietore	727887	5145740	1180	27.1	5.3	93.4	6.0	813	Sedim. carbonate	Bidussi M., Capozzi F.
34	Veneto (BL)	Vigo di Cadore	778437	5155969	1540	22.6	4.5	94.6	3.6	1019	Sedim. clastic	Capozzi F., Panepinto F.
35	Friuli Venezia Giulia (UD)	Prato Carnico	780331	5156611	1295	22.4	4.7	94.5	5.2	998	Sedim. clastic	Capozzi F., Panepinto F.
36	Friuli Venezia Giulia (UD)	Ampezzo	782515	5149030	1460	21.8	5.0	94.2	4.3	1020	Sedim. carbonate	Capozzi F., Panepinto F.
37	Friuli Venezia Giulia (UD)	Forni di Sotto	782592	5149904	1275	21.7	4.9	94.3	4.9	1011	Sedim. carbonate	Capozzi F., Panepinto F.
38	Friuli Venezia Giulia (UD)	Ampezzo	782896	5148406	1650	21.9	5.0	94.1	3.2	1046	Sedim. carbonate	Capozzi F., Panepinto F.
39	Friuli Venezia Giulia (UD)	Ampezzo	783063	5147585	1970	22.2	5.1	94.1	2.3	1078	Sedim. carbonate	Capozzi F., Panepinto F.
40	Friuli Venezia Giulia (UD)	Ampezzo	783186	5148045	1850	22.5	5.1	94.1	3.0	1050	Sedim. carbonate	Capozzi F., Panepinto F.
41	Friuli Venezia Giulia (UD)	Tarvisio	860311	5156642	1830	13.6	5.4	93.3	2.6	1266	Sedim. carbonate	Martellos S.
42	Toscana (LU)	San Romano in Garfagnana	608580	4895302	1230	50.7	17.6	80.3	7.4	878	Sedim. carbonate	Benesperi R.
43	Toscana (PT)	Abetone	632906	4887038	1315	54.9	18.6	79.6	6.1	859	Sedim. carbonate	Benesperi R
44	Toscana (PT)	Abetone	633384	4889793	1425	56.3	20.8	77.4	6.8	873	Sedim. carbonate	Benesperi R
45	Toscana (FI)	Reggello	706379	4846165	1130	196.9	34.8	60.9	8.2	907	Sedim. carbonate	Benesperi R
46	Toscana (AR)	Poppi	726310	4851715	960	38.5	21.3	77.3	9.1	903	Sedim. carbonate	Benesperi R
47	Lazio (VT)	Soriano nel Cimino	763440	4700006	1060	124.1	76.4	20.5	10.8	707	Igneous extrusive	Ravera S.
48	Umbria (TR)	Polino	818942	4721156	1400	113.3	28.7	68.3	7.3	818	Sedim. carbonate	Ravera S.
49	Lazio (RI)	Leonessa	829212	4711427	1620	45.1	25.5	73.2	6.4	814	Sedim. carbonate	Ravera S.
50	Lazio (FR)	Filettino	862329	4649914	1770	37.2	32.9	64.4	6.9	805	Sedim. carbonate	Ravera S.
51	Lazio (FR)	Filettino	862883	4648429	1600	44.7	34.4	62.9	6.5	803	Sedim. carbonate	Ravera S.
52	Campania (AV)	Bagnoli Irpino	1016725	4532218	1490	146.1	45.7	50.6	7.6	708	Sedim. carbonate	Capozzi F.
53	Basilicata (PZ)	Sasso di Castalda	1071257	4504090	1615	94.1	45.4	52.3	6.9	775	Sedim. carbonate	Potenza G., Romano A.
54	Basilicata (PZ)	Abriola	1071592	4503849	1615	93.6	45.3	52.4	6.9	776	Sedim. carbonate	Potenza G., Romano A.
55	Calabria (CS)	Morano Calabro	1103640	4434506	1335	60.9	30.9	67.2	8.9	856	Sedim. carbonate	Puntillo D.
56	Calabria (CS)	Morano Calabro	1114439	4442288	2125	52.2	34.5	64.3	4.4	894	Sedim. carbonate	Puntillo D.
57	Calabria (CS)	Celico	1138732	4381258	1430	137.7	32.9	63.7	9.1	865	Metamorphic	Puntillo D.
58	Calabria (CS)	Spezzano Piccolo	1141751	4378020	1650	136.4	28.5	68.1	7.7	848	Metamorphic	Puntillo D.
59	Calabria (CS)	Spezzano della Sila	1144011	4379400	1440	127.9	26.7	70.2	9.1	865	Igneous intrusive	Puntillo D.
60	Calabria (CS)	Celico	1149217	4388410	1130	77.7	28.8	69.6	10.5	869	Igneous intrusive	Puntillo D.
61	Calabria (CZ)	Taverna	1158747	4355740	1600	87.2	32.4	65.4	8.1	874	Metamorphic	Puntillo D.
62	Sicilia (CT)	Randazzo	1016912	4210511	960	51.5	34.0	64.6	12.5	609	Sedim. clastic	Carasci A., Cataldo D.

Supplementary Table S2. Results of the literature review on element content in the lichen *Pseudevernia furfuracea*. Data refer to country (ISO 3166-1 codes), study type (A: active biomonitoring, P: passive biomonitoring, O: other), number of sampling sites in the 62 reference studies, and set of targeted chemical elements. The indication of number of collection (c) and exposure (e) sites is also reported (in case of passive biomonitoring, samples are considered life-span exposed and the number of sites is placed in column 'e'; missing data for type A indicate lack of information).

Reference	Country	Type	Sites		Elements
			c	e	
Adamo et al. 2003	IT	A	1	11	Al, As, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Ni, Pb, Ti, V, Zn
Adamo et al. 2007	IT	A	1	2	Al, Ca, Cd, Cr, Cu, Fe, Hg, K, Mg, Mn, Na, Ni, Pb, V, Zn
Adamo et al. 2008	IT	A	1		Al, C, Ca, Cd, Cr, Cu, Fe, Hg, K, Mg, Mn, N, Na, Ni, Pb, S, V, Zn
Aksoy et al. 2010	TR	A		1	Ca, Cd, Co, Cr, Cu, K, Pb, Sr, Ti, Zn
Aslan et al. 2004	TR	P		1	Ba, Ca, Fe, K, Sr, Ti
Aslan et al. 2006	TR	P		1	Ba, Ca, Fe, K, Ti
Aslan et al. 2013	TR	A		4	Al, Cd, Cr, Cu, Ni, Pb
Bari et al. 2001	IT	A	1	1	Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn
Basile et al. 2008	IT	A	1	1	Al, As, Cd, Cr, Cu, Fe, Mn, Pb, V, Zn
Bergamaschi et al. 2007	IT	A	1	1	Al, As, Br, Ca, Cd, Ce, Cl, Co, Cr, Cs, Cu, Fe, Hg, I, K, La, Mg, Mn, Ni, Pb, Rb, Sb, Sc, Se, Sm, Th, Ti, V, Zn
Brienza et al. 2009	IT	P		6	Al, As, Cd, Cr, Cu, Ni, Pb, Zn
Bylińska, 1996	PL	P		1	La, Ti, V
Calliari et al. 1995	IT	A	1	2	Ca, Cu, Fe, K, Mg, Mn, Pb, S, Zn
Cansaran-Duman et al. 2009	TR	P		11	Cr, Fe, Mn, Pb, Zn
Cansaran-Duman and Aras, 2012	TR	P		11	Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn
Cansaran-Duman et al. 2012	TR	A	1	10	Cd, Cu, Mn, Ni, Pb, Zn
Carasci and Cataldo, 2016	IT	A	1	3	Al, As, Ba, Be, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, Ti, V, Zn
Cardarelli et al. 1993	IT	A	1	5	Al, Cu, Mn, Pb, Zn
Cicek et al. 2008	TR	A	1	4	Cr, Cu, Fe, Ni, Pb, Zn
Cisaro et al. 2005	IT	A	1		Al, Cd, Cr, Cu, Fe, Mn, Ni, Pb, V, Zn
Corapi et al. 2014	IT	A	1	1	Al, As, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Pb, Sb, Se, V, Zn
Culicov and Yurukova, 2006	BG	A	1	1	Al, As, Au, Ba, Br, Ca, Cd, Cl, Co, Cr, Cs, Cu, Fe, Gd, Hf, I, K, La, Mg, Mn, Na, Ni, Rb, Sb, Sc, Sm, Sr, Ta, Tb, Th, Ti, U, V, Zn
Eğilli et al. 2003	TR	P		1	As, Co, Cr, Fe, Hg, Mn, Sb, Se, Zn
Folkeson, 1979	SE	P		1	Cd, Cu, Fe, Ni, Pb, Zn
Gallo et al. 2014	IT	A	1	10	Al, Ca, Fe, K, Mg, Mn, Na, V
Garty and Amman, 1987	CH	P		17	Cr, Cu, Fe, Mn, Ni, Pb, Zn
Giordano et al. 2005	IT	A	1	11	Al, As, Cd, Co, Cr, Cu, Fe, Mo, Ni, Pb, Ti, V, Zn
Giordano et al. 2009	IT	A	1	1	Al, Ba, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Mo, Na, Ni, Pb, Sr, Ti, V, Zn
Giordano et al. 2013	IT	A	1	4	Al, As, Ba, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Mo, Ni, Pb, Ti, V, Zn
Griselli et al. 2002	IT	A	1		Al, Cd, Cr, Cu, Fe, Mn, Ni, Pb, V, Zn
Guidotti et al. 2009	IT	A	1	5	Cd, Cr, Cu, Ni, Pb, Zn
Jozic et al. 2009	AT	A	1	5	Cd, Cu, Pb, Zn
Loppi et al. 2003	IT, GR, RS, ME	P		21	U
Loppi, 2014	IT	P		2	Al, As, Ca, Cd, Ce, Co, Cr, Cs, Cu, Dy, Er, Eu, Fe, Gd, Ho, Ir, K, La, Lu, Mg, Mn, Na, Nd, Ni, Pb, Pd, Pr, Pt, Rh, Ru, S, Sb, Sc, Sm, Sr, Tb, Tm, U, V, Yb, Zn
Lounamaa, 1965	FI	P		2	Fe, Mn, Zn
Lucadamo et al. 2015	IT	A	1	32	Al, As, Co, Cr, Cu, Mn, Mo, Sb, Sn, Ti, V, Zn
Magnani, 1998	IT	A	1	12	Cd, Cr, Mn, Ni, Pb, V, Zn
Malaspina et al. 2014	IT	A	1	1	Al, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, Sr, V, Zn
Mlakar et al. 2011	SI	A	1	8	Hg
Oztetik and Cicek, 2011	TR	A	1	3	Al, B, Ca, Cu, Fe, K, Mg, Mn, Ni, P, Pb, S, Zn
Pantelică et al. 2005	IT	A	1		Ca, Cd, Cr, Cu, Fe, K, Mn, Ni, Pb, V, Zn
Petrova et al. 2015	BG	A	1	1	Al, As, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Na, Ni, P, Pb, S, Sr, U, V, Zn
Piervittori, 1998	IT	A	1	2	Cd, Cr, Cu, Mn, Pb, Zn
Pirintsos et al. 2004	GR	A	1	8	Al, Cd, Cr, Cu, Fe, Ni, Pb, V, Zn
Pirintsos et al. 2006	GR	A	1	2	Al, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn
Protano et al. 2014	IT	A	1	11	As, Cd, Cr, Cu, Ni, Pb, Zn
Ricchiardone and Bari, 2003	IT	A	1		Cd, Cr, Pb, Zn
Roos-Barraclough et al. 2002	CH	O		1	Hg
Rossbach and Lambrecht, 2006	DE	P		1	As, Ba, Cd, Co, Cu, Fe, Hg, Ni, Pb, S, Se, Tl, Zn
Saib, 2014	DZ	A	1	40	Pb
Sorbo et al. 2008	IT	A	1	18	Al, As, Cd, Cr, Cu, Fe, Mn, Pb, V, Zn
Spagnuolo et al. 2011	IT	A	1	1	Ca, Cu, K, Mg, Pb, Zn
Stratis et al. 1999	GR	P		2	Cd, Cu, Pb, Zn
Takala and Olkkonen, 1985	FI	P		n.a.	Ti
Takala et al. 1985	FI	P		n.a.	S
Takala et al. 1994	FI	P		n.a.	Fe, S, Ti
Takala et al. 1998	FI	P		n.a.	Zn
Tretiach et al. 2011	IT	A	1	31	Al, As, Ca, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Sb, Sn, V, Zn
Vingiani et al. 2004	IT	A	1	13	C, N, S
Vingiani et al. 2015	IT, GB	A	1	8	Cd, Cr, Cu, Fe, Ni, Pb, V, Zn
Yildiz et al. 2008	TR	A	1	27	Cu, Mn, Ni, Pb, Zn
Yildiz et al. 2011	TR	A	1	10	Cd, Cu, Mn, Ni, Pb, Zn

Supplementary Table S3. Counts of data on element content in the lichen *Pseudevernia furfuracea* included in the *background* (BG), passive (P) and *transplant* (T) datasets. In the case of BG dataset, counts are separately reported for data from field sampling in Italy and for previous reviewed studies. Numbers in brackets refer to counts of values from field samples resulted below the analytical limit of detection. Outliers refer to the number of data cleaned out from the dataset (see main text) and excluded from the total counts. Elements not selected for data analysis due to insufficient sample size after data cleaning are marked with asterisk.

Element	BG				P	T	Total counts
	Field	Literature	Outliers	Selected			
Ag	62		1	61			61
Al	62	20	1	81	8	208	297
As	62(12)	2	1	63	10	145	218
Au	62		1	61		1	62
B*	62(35)			0		3	3
Ba	62	2	1	63	1	29	93
Be*	62(62)			0		9	9
Bi	62(2)		0	62			62
Br*						6	6
Ca	62	12		74	4	97	175
Cd	62	26	1	87	25	272	384
Ce	62		1	61	2	5	68
Cl*						6	6
Co	62	4	1	65	4	110	179
Cr	62	19	1	80	48	263	391
Cs	62		4	58	2	6	66
Cu	62	29		91	42	329	462
Dy*					2		2
Er*					2		2
Eu*					2		2
Fe	62	17		79	57	204	340
Ga*	62(32)			0			0
Gd*					2	1	3
Ge	62(17)		1	61			61
Hf	62			62		1	63
Hg	62	12		74	2	59	135
Ho*					2		2
I*						6	6
In*	62(62)			0			0
K	62	12		74	4	91	169
La	62		1	61	5	6	72
Li	62			62			62
Lu*					2		2
Mg	62	11	1	72	2	91	165
Mn	62	29	1	90	43	300	433
Mo	62	3		65		91	156
Na	62	11		73	2	33	108
Nb	62			62			62
Nd*					2		2
Ni	62	26	1	87	38	253	378
P	62	1		63		4	67
Pb	62	30	7	85	53	336	474
Pd	62(25)		3	59	2		61
Pr*					2		2
Pt*	62(54)			0	2		2
Rb	62			62		6	68
Re*	62(52)			0			0
Rh*					2		2

Supplementary Table S3 (continued)

Element	BG				P	T	Total counts
	Field	Literature	Outliers	Selected			
Ru*					2		2
S	62	3		65	11	49	125
Sb	62	1		63	3	53	119
Sc	62			62	2	6	70
Se	62		3	59	2	17	78
Sm*					2	6	8
Sn	62	1	1	62		38	100
Sr	62	3	1	64	2	6	72
Ta*	62(33)			0		1	1
Tb*					2	1	3
Te*	62(62)			0			0
Th	62			62		6	68
Ti	62	3	1	64	21	88	173
Tl*	62(51)			0	1		1
Tm*					2		2
U	62(6)	1		63	23	1	87
V	62(19)	16	5	73	5	181	259
W*	62(54)			0			0
Y	62		1	61			61
Yb*					2		2
Zn	62	30		92	59	336	487
Zr	62			62			62
Total	**2666**	**324**	**40**	**2950**	**513**	**3760**	**7223**

Supplementary Table S4. Differences in environmental conditions (Kruskal-Wallis ANOVA, H and associated *p*-value) among clusters of sites with similar *Pseudevernia furfuracea* element content. Data refer to median and interquartile range of environmental descriptors calculated over the sites of each cluster (number of sites in brackets). Different letters indicate significantly different groups within each row (Dunn's post hoc test, $p < 0.05$).

Environmental descriptors	Clusters of sites			K-W ANOVA	
	I (n=22)	II (n=20)	III (n=20)	H	*p*
Anthropization					
Population density (km^{-2})	68.2 **ab** (57.6 ÷ 75.3)	30.0 **a** (22.3 ÷ 55.6)	90.4 **b** (51.8 ÷ 132.1)	15.4	*0.0005*
Land use					
Agricultural areas (% coverage)	10.2 **a** (9.2 ÷ 13.2)	6.8 **a** (5.1 ÷ 18.1)	32.7 **b** (27.6 ÷ 34.6)	28.4	*< 10^{-4}*
Forests and seminatural areas (%)	87.9 **b** (85.7 ÷ 89.1)	91.9 **b** (79.8 ÷ 94.1)	65.0 **a** (61.9 ÷ 69.9)	29.2	*< 10^{-4}*
Climate					
Annual temperature (°C)	3.6 **a** (2.0 ÷ 5.5)	4.0 **a** (3.2 ÷ 6.1)	7.7 **b** (6.7 ÷ 9.0)	26.8	*< 10^{-4}*
Minimum temperature of coldest month (°C)	-7.4 **a** (-8.4 ÷ -5.9)	-8.7 **a** (-9.4 ÷ -6.5)	-2.4 **b** (-3.5 ÷ -0.4)	31.0	*< 10^{-4}*
Maximum temperature of warmest month (°C)	16.3 **a** (14.9 ÷ 20.0)	17.9 **a** (17.1 ÷ 19.7)	20.5 **b** (19.7 ÷ 21.4)	16.2	*0.0003*
Annual precipitation (mm)	1003 **b** (962 ÷ 1065)	915 **b** (867 ÷ 1033)	833 **a** (775 ÷ 872)	22.2	*< 10^{-4}*
Precipitation of wettest month (mm)	128 **b** (122 ÷ 134)	125 **b** (108 ÷ 132)	104 **a** (98 ÷ 116)	18.3	*0.0001*
Precipitation of driest month (mm)	48 **b** (42 ÷ 61)	45 **b** (36 ÷ 49)	31 **a** (23 ÷ 44)	16.3	*0.0003*
Lithology					
Igneous rocks (% coverage)	0 **a** (0 ÷ 0)	0 **a** (0 ÷ 0)	0 **a** (0 ÷ 0)	4.3	0.1160
Metamorphic rocks (%)	89 **b** (0 ÷ 100)	0 **a** (0 ÷ 0)	0 **ab** (0 ÷ 43)	13.2	*0.0014*
Sedimentary carbonate rocks (%)	0 **a** (0 ÷ 0)	100 **b** (97 ÷ 100)	97 **b** (0 ÷ 100)	25.3	*< 10^{-4}*
Sedimentary clastic rocks (%)	0 **a** (0 ÷ 22)	0 **a** (0 ÷ 0)	0 **a** (0 ÷ 0)	2.7	0.2601

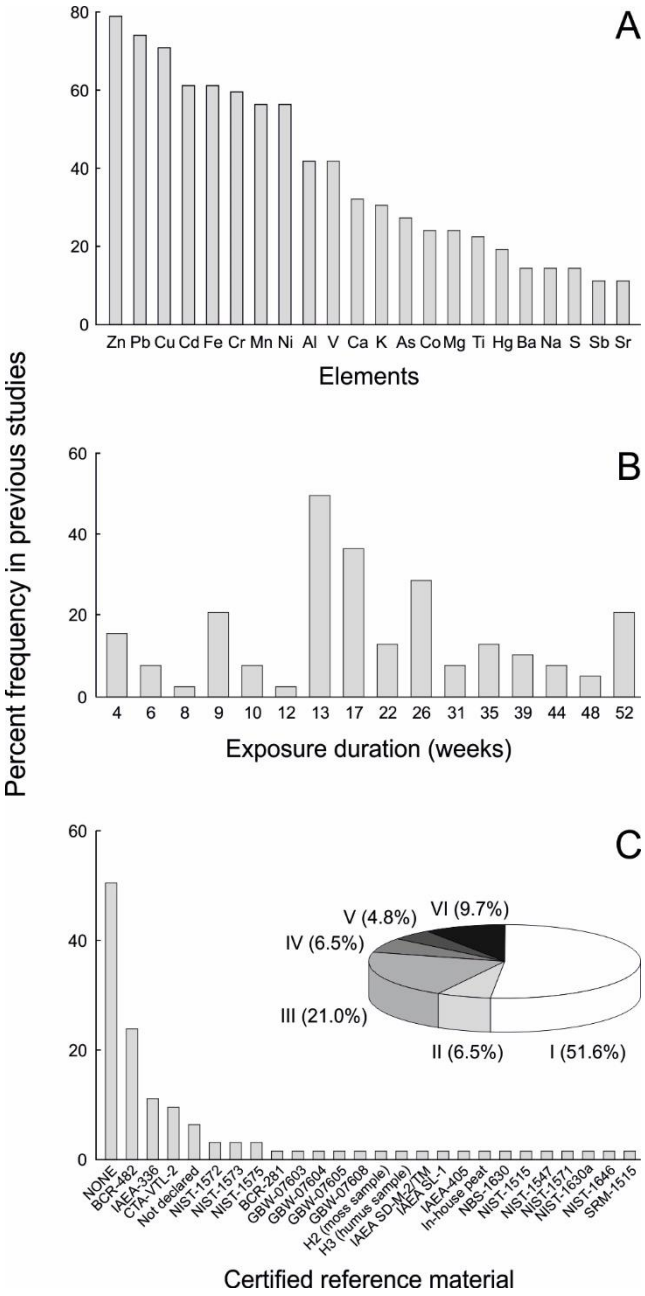
Supplementary Table S5. *Pseudevernia furfuracea* element content in Italy. Data refer to descriptive statistics (counts, mean ± standard deviation, 95% confidence interval, median, interquartile range, minima and maxima) for 43 elements from the field data included in the background (BG) dataset.

Element	Mean ± SD	C.I. 95%	Median	q1 - q3	Min	Max
Ag	0.020 ± 0.007	0.018 ÷ 0.022	0.019	0.014 ÷ 0.023	0.009	0.044
Al	461 ± 228	403 ÷ 519	390	300 ÷ 550	200	1200
As	0.203 ± 0.095	0.179 ÷ 0.227	0.179	0.134 ÷ 0.26	0.090	0.525
Au	0.122 ± 0.080	0.102 ÷ 0.143	0.106	0.062 ÷ 0.162	0.010	0.322
Ba	12.0 ± 5.6	10.6 ÷ 13.4	10.6	8.1 ÷ 13.6	4.1	29.1
Bi	0.046 ± 0.021	0.040 ÷ 0.051	0.042	0.030 ÷ 0.060	0.019	0.121
Ca	7178 ± 4161	6122 ÷ 8235	5563	4620 ÷ 9060	2423	20780
Cd	0.145 ± 0.051	0.132 ÷ 0.158	0.140	0.100 ÷ 0.169	0.068	0.313
Ce	1.06 ± 0.59	0.91 ÷ 1.22	0.87	0.64 ÷ 1.43	0.36	3.10
Co	0.256 ± 0.096	0.231 ÷ 0.281	0.243	0.170 ÷ 0.314	0.120	0.505
Cr	3.01 ± 1.02	2.75 ÷ 3.27	2.78	2.52 ÷ 3.17	2.00	9.54
Cs	0.104 ± 0.053	0.090 ÷ 0.118	0.089	0.067 ÷ 0.127	0.043	0.298
Cu	5.57 ± 2.15	5.02 ÷ 6.11	5.00	3.86 ÷ 6.81	2.86	12.51
Fe	547 ± 250	483 ÷ 610	489	364 ÷ 691	218	1385
Ge	0.013 ± 0.004	0.012 ÷ 0.014	0.012	0.011 ÷ 0.015	0.009	0.024
Hf	0.054 ± 0.026	0.048 ÷ 0.061	0.051	0.032 ÷ 0.069	0.020	0.131
Hg	0.207 ± 0.056	0.193 ÷ 0.221	0.200	0.169 ÷ 0.251	0.094	0.355
K	3404 ± 546	3266 ÷ 3543	3361	3040 ÷ 3760	2150	4720
La	0.469 ± 0.267	0.401 ÷ 0.538	0.383	0.273 ÷ 0.664	0.138	1.374
Li	0.345 ± 0.167	0.302 ÷ 0.387	0.307	0.221 ÷ 0.444	0.126	0.948
Mg	774 ± 175	729 ÷ 819	728	658 ÷ 864	467	1248
Mn	58.7 ± 31.4	50.7 ÷ 66.7	49.3	36.2 ÷ 71.8	16.3	175.7
Mo	0.254 ± 0.145	0.217 ÷ 0.291	0.236	0.130 ÷ 0.335	0.090	0.790
Na	84.1 ± 70.7	66.1 ÷ 102.0	42.5	30.0 ÷ 152.5	20.0	273.8
Nb	0.042 ± 0.024	0.036 ÷ 0.048	0.035	0.026 ÷ 0.053	0.012	0.126
Ni	1.31 ± 0.61	1.15 ÷ 1.47	1.17	0.94 ÷ 1.53	0.48	3.54
P	565 ± 160	524 ÷ 607	536	463 ÷ 633	308	1060
Pb	2.98 ± 1.20	2.67 ÷ 3.29	2.65	2.20 ÷ 3.66	0.80	7.83
Pd	0.0027 ± 0.001	0.0024 ÷ 0.0029	0.0023	0.0019 ÷ 0.003	0.0019	0.0062
Rb	11.7 ± 7.1	9.9 ÷ 13.5	10.6	6.1 ÷ 14.9	2.3	33.8
S	1515 ± 217	1460 ÷ 1570	1530	1350 ÷ 1644	1013	2260
Sb	0.093 ± 0.052	0.080 ÷ 0.106	0.083	0.054 ÷ 0.118	0.029	0.284
Sc	0.358 ± 0.056	0.344 ÷ 0.372	0.350	0.320 ÷ 0.390	0.240	0.500
Se	0.276 ± 0.095	0.251 ÷ 0.301	0.267	0.217 ÷ 0.3	0.096	0.550
Sn	0.337 ± 0.166	0.295 ÷ 0.380	0.302	0.217 ÷ 0.414	0.108	0.945
Sr	15.1 ± 8.2	13.0 ÷ 17.2	11.5	9.5 ÷ 19.3	5.7	46.0
Th	0.103 ± 0.063	0.088 ÷ 0.119	0.089	0.056 ÷ 0.138	0.030	0.340
Ti	11.3 ± 4.7	10.1 ÷ 12.5	10.6	7.8 ÷ 13.9	4.2	26.6
U	0.02 ± 0.011	0.017 ÷ 0.023	0.020	0.011 ÷ 0.024	0.009	0.056
V	2.18 ± 0.38	2.08 ÷ 2.27	1.98	1.92 ÷ 2.35	1.90	3.20
Y	0.551 ± 0.258	0.485 ÷ 0.617	0.458	0.350 ÷ 0.719	0.200	1.286
Zn	36.8 ± 16.2	32.7 ÷ 40.9	35.3	25.5 ÷ 41.5	14.2	83.6
Zr	2.28 ± 1.01	2.03 ÷ 2.54	2.33	1.44 ÷ 2.9	0.94	5.31

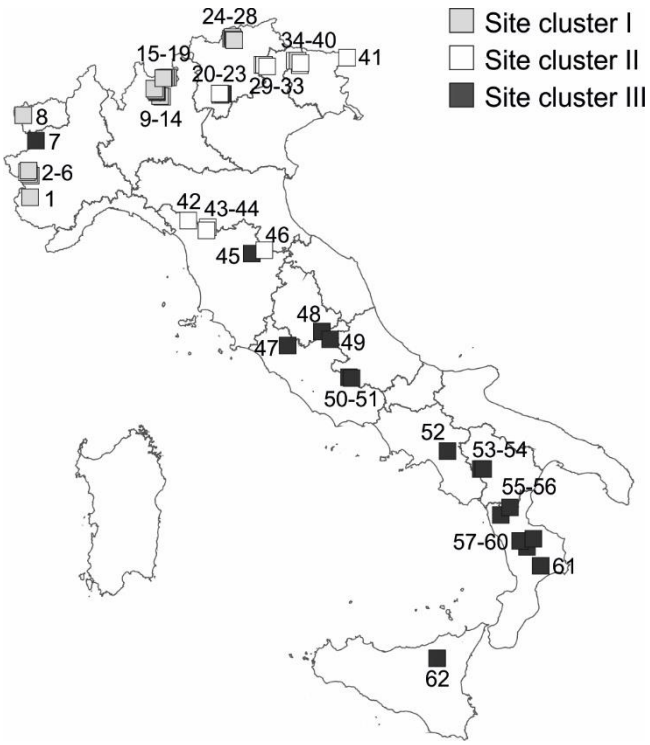
Supplementary Table S6. Results of Principal Component Regression (PCR) modelling of *Pseudevernia furfuracea* element content in Italian samples as related to environmental conditions at the collection sites. Top: correlation between environmental descriptors and PCs (variance explained by each PC in brackets). Bottom: standardized coefficients (β) of the PCs in the PCR models of 43 chemical elements ordered according to CA, limited to statistically significant values ($p < 0.05$).

		PC 1 (45.3%)	**PC 2** (14.3%)	**PC 3** (10.2%)	**PC 4** (9.7%)	**PC 5** (7.7%)	**PC 6** (6.0%)	**PC 7** (3.4%)	**PC 8** (2.2%)	**PC 9** (1.1%)	**PC 10** (0.17%)	**PC 11** (0.003%)	**PC 12** (0.002%)
Environmental descriptors		**Correlation coefficients**											
Population density (km^{-2})		-0.54	0.19	-0.45	0.08	0.26	-0.50	0.37	-0.15	-0.01	0.01	0.00	0.00
Agricultural areas (%)		-0.86	0.18	-0.13	-0.01	0.35	0.06	-0.28	0.01	-0.09	0.00	0.01	0.01
Forest and seminatural areas (%)		0.86	-0.17	0.16	-0.01	-0.36	-0.02	0.24	-0.01	0.09	0.00	0.01	0.01
Annual temperature (°C)		-0.88	0.21	0.33	0.01	-0.15	0.18	0.12	-0.12	0.02	0.00	0.01	-0.01
Warmest month temperature (°C)		-0.79	0.10	0.41	0.07	-0.22	0.21	0.24	-0.05	-0.20	0.01	-0.01	0.00
Coldest month temperature (°C)		-0.88	0.29	0.16	-0.04	0.04	0.13	-0.06	-0.16	0.27	0.01	-0.01	0.00
Annual precipitation (mm)		0.81	0.33	0.09	0.01	0.36	0.22	0.08	-0.19	-0.03	-0.10	0.00	0.00
Precipitation of wettest month (mm)		0.86	0.09	-0.02	-0.28	-0.04	0.02	-0.16	-0.36	-0.07	0.07	0.00	0.00
Precipitation of driest month (mm)		0.63	0.34	0.12	0.22	0.52	0.31	0.20	0.16	0.03	0.07	0.00	0.00
Igneous rocks (%)		-0.14	0.26	-0.33	-0.86	-0.06	0.19	0.13	0.11	0.00	0.00	0.00	0.00
Metamorphic rocks (%)		0.24	0.70	-0.21	0.53	-0.33	-0.03	-0.10	0.01	-0.01	0.00	0.00	0.00
Carbonate rocks (%)		-0.23	-0.90	-0.03	0.20	0.23	0.17	0.05	-0.11	0.01	0.00	0.00	0.00
Clastic rocks (%)		0.17	0.15	0.78	-0.24	0.22	-0.48	-0.06	0.06	0.00	0.00	0.00	0.00
Element group	**Element**	**β coefficients**											
1	Ag	-	-	-	-	-	-	-	-	-	0.355	-	-
	Au	-	-	-	-	-	-	-	-	-	-	-	-
	Bi	-	0.452	-	-	-	-	-	0.510	-	-	-	-
	Cs	-	-	-0.319	-	-	-	-		-	-	-	-
	Cu	-	-	-	-	-	-0.429	-	0.305	-	-	-	-
	Mo	0.543	0.455	-	-	-	-0.367	-	0.373	-	0.301	-	0.256
	Rb	-	-	-	-	-	-	-		-	-	-	-
	Sb	-	-	-	0.314	-	-	-	0.373	-0.316	-	-	-
	Sn	-	0.319	-	0.320	-	-	-	0.537	-0.333	-	-	-
	Zn	0.498	0.280	-0.319	-	-0.289	-0.377	-	-	-	-	-	-
2	Ba	-	-	-	-	-	-	-	-	-	-	-	-
	Co	-0.415	0.328	-	-0.272	-0.366	0.297	-	0.448	0.513	-0.497	-	-
	Cr	-	-	-	-0.372	-	-	-	-	-	-	-	-
	K	-0.550	-	-	-	-	-	-0.298	-	-	-	-	-
	Mg	-0.520	-	-	-	-	-	-	-	-	-	-	-
	Mn	-	-	-	-	-	-	-	-	-	-	-	-
	Ni	-	0.459	-	-0.406	0.242	-	0.223	0.413	-	-	-	0.268
	P	-	0.370	0.320	-	0.381	-	-	-	-	-	-	-
	Pd	-	-	-	-	-	-	-	-	-	-	-	-
3	Al	-0.710	-	-	-	-	-	-	-	-	-	-	-
	Ca	-0.653	-	-	-	-	-	-0.240	-	0.293	-0.253	-	-
	Ce	-0.801	0.247	-	-	0.230	-	-0.414	-	-	-	-0.219	-0.335
	Fe	-0.552	-	-	-	-	-	-0.319	-	-	-0.308	-0.326	-
	Hf	-0.447	0.256	-	-	-	-	-0.297	-	-	-0.275	-	-
	La	-0.810	0.215	-	-	-	-	-0.246	-	-	-	-	-
	Li	-0.560	0.344	-	-	-	-	-0.241	-	-	-0.315	-	-
	Na	-0.734	-	-	-	0.224	0.205	-	-0.286	0.173	0.208	-	-
	Nb	-0.453	-	-	-	-	-	-0.373	-	-	-	-	-
	Sr	-0.833	-	0.179	-	-	-	-0.317	-0.202	0.223	-0.178	-	-
	Th	-0.615	0.438	-	-	-	-	-0.243	-	-	-	-	-
	Ti	-0.606	0.365	-	-	-	-	-	-	-	-	-	-
	U	-0.507	-	-	-	-	-	-0.392	-	-	-	-	-
	Y	-0.660	0.345	-	-	-	-	-0.357	-	-	-0.387	-	-
	Zr	-0.495	0.337	-	-	-	-	-	-	-	-	-	-
4	As	-	-	-	-	-	-	-	-	-	-	-	-
	Cd	-0.446	-	-	0.289	-	-	-0.474	-	-	-	-	-
	Ge	-0.454	-	-	-	-	-	-0.413	-	-	-	-	-
	Hg	-	-	-	-	-	-	-	-	-	-	-	-
	Pb	-	-	-	-	-	-	-	-	-	-	-	-
	S	-0.430	-	-	-	-	-	-	-	-	-	-	-
	Sc	-0.593	-	-	-	-	-	-	-	-	-	-	-
	Se	-0.607	-	-	-	0.415	-	-	-	-	-	-	-
	V	-0.391	-	-	-	-	-	-	-	-	-0.285	-	0.391

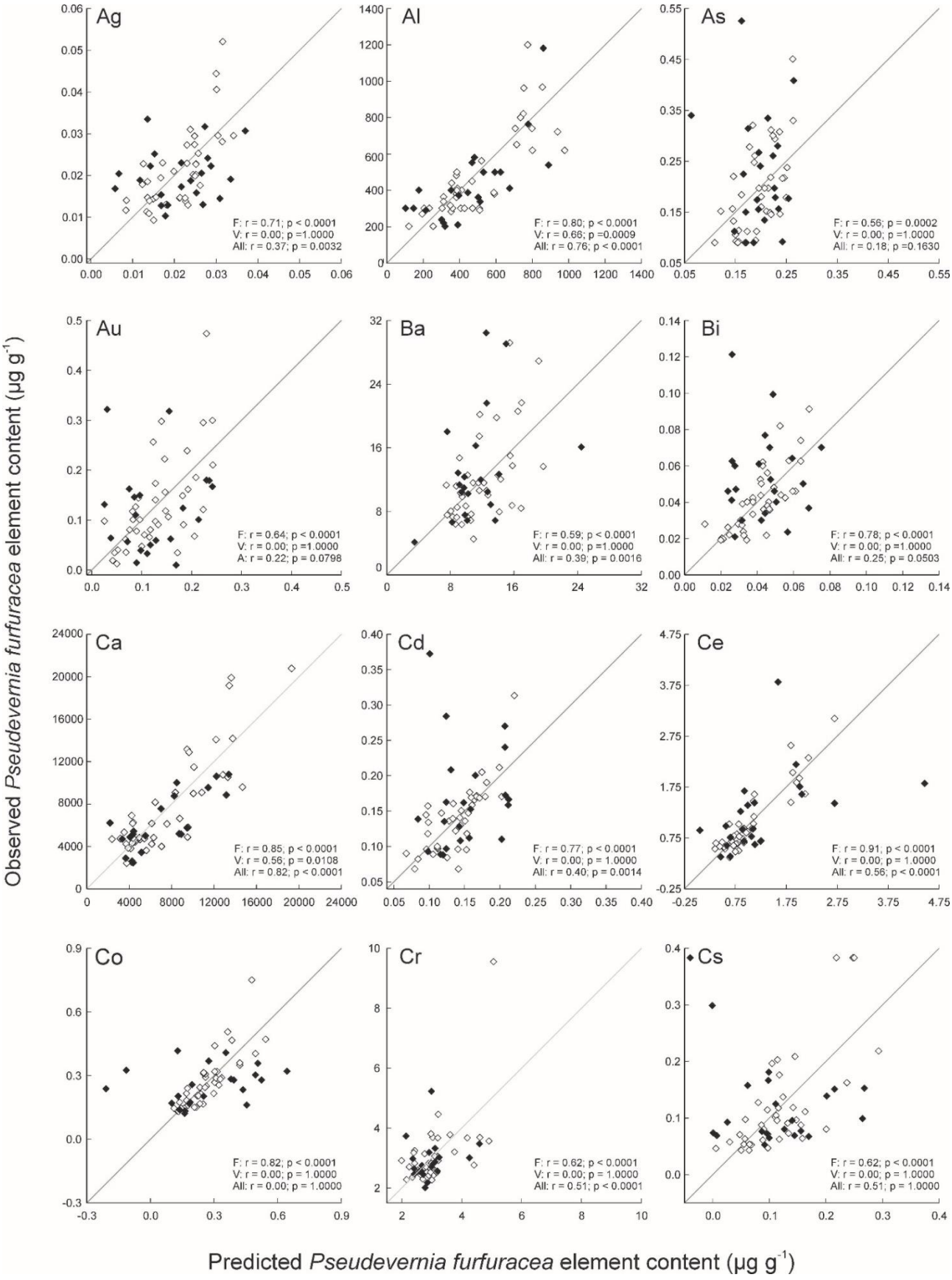
SUPPLEMENTARY FIGURES S1-S3



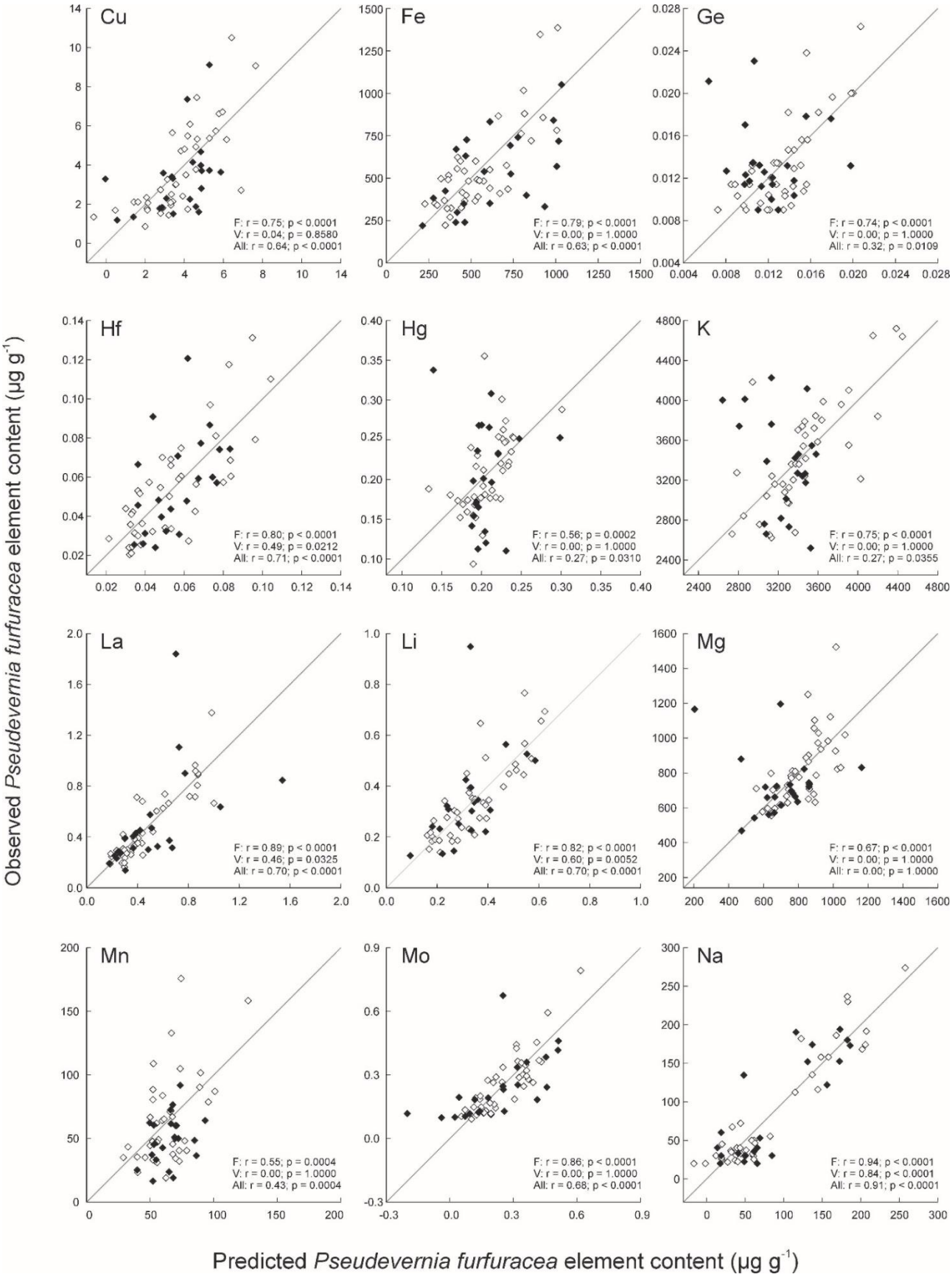
Supplementary Figure S1. Overview of previous studies on element content in *Pseudevernia furfuracea*. Data refer to (A) chemical elements targeted in at least 10% of the reviewed studies, (B) the range of exposure duration in lichen transplants, i.e. bags with selected terminal lobes (68% of the cases), artificial twigs carrying entire thalli (21%), other methods (11%), and (C) the frequency of different CRMs (lichen materials: BCR 482 '*Pseudevernia furfuracea'*, Quevauviller et al. 1996; IAEA-336 '*Evernia prunastri*', Stone et al. 1995; Schmeling et al. 2007) and options used for reporting QC information (pie-chart in the inset). Inset labels refer to the options reported in Table 1.



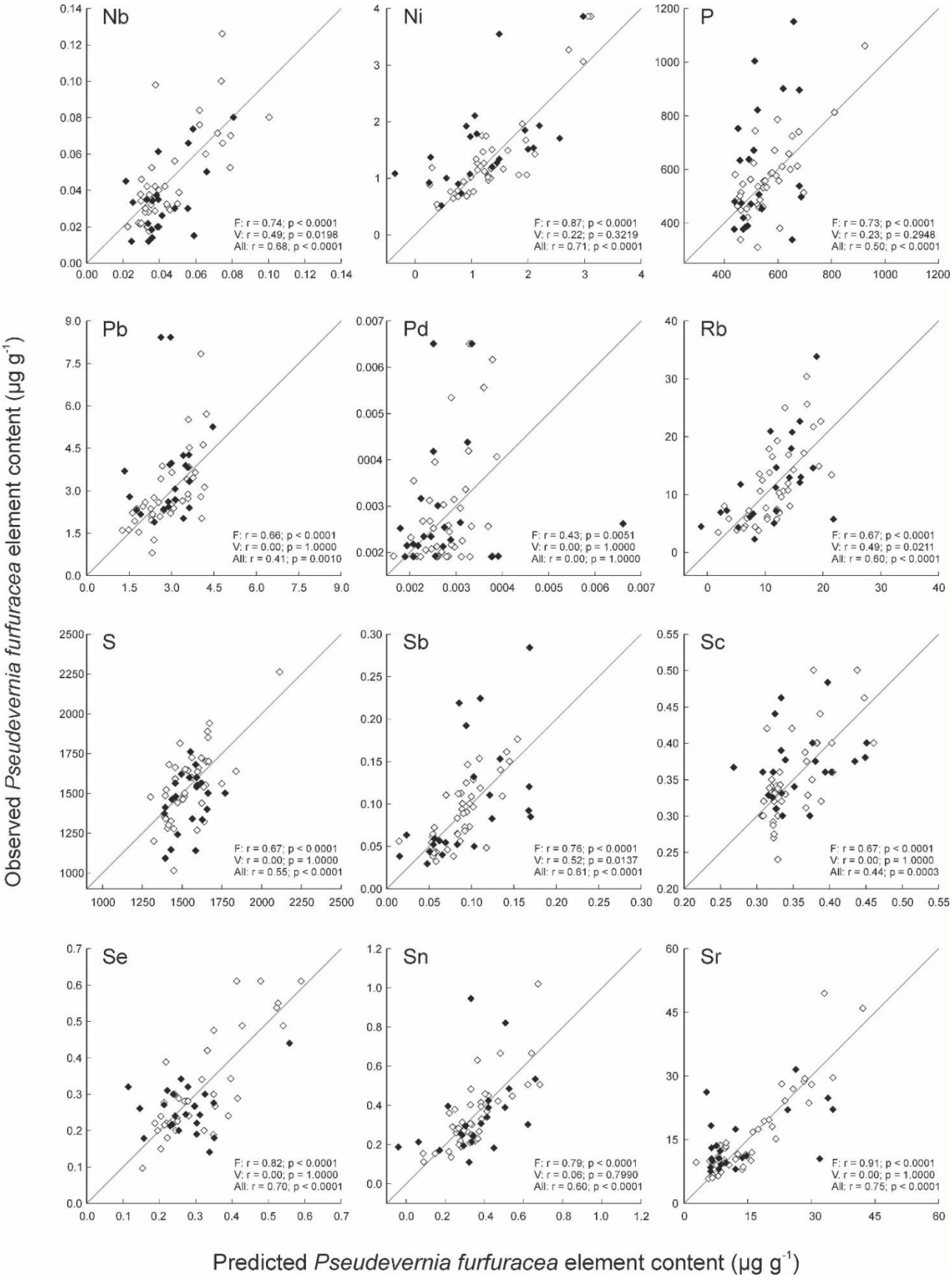
Supplementary Figure S2. Map of field sites (ID codes and details in Supplementary Table S1) symbolized according to CA results (Fig. 3).



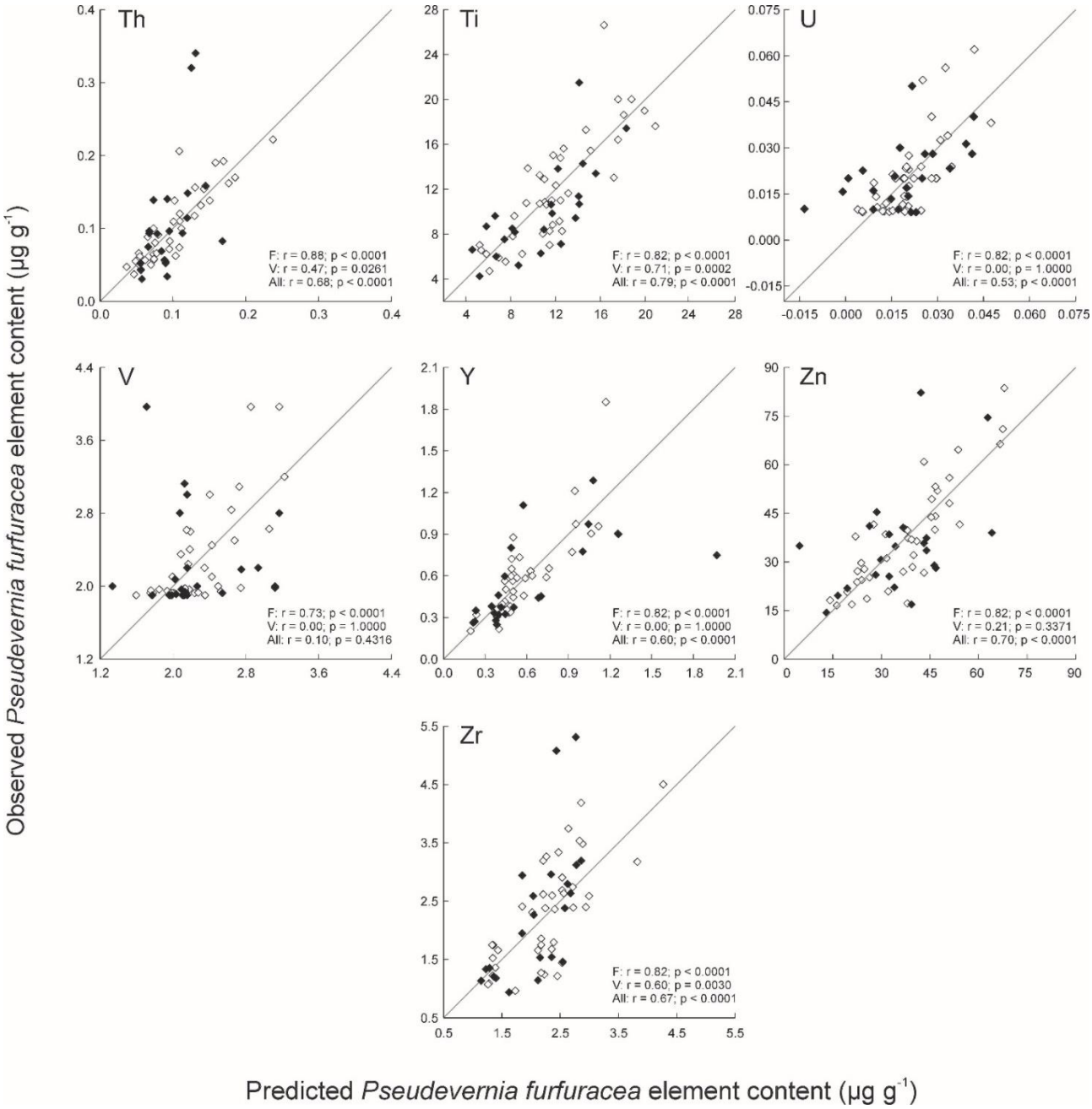
Supplementary Figure S3. Results of PCR modelling of *Pseudevernia furfuracea* element content in Italy for Ag, Al, As, Au, Ba, Bi, Ca, Cd, Ce, Co, Cr, Cs. For each element, a scatterplot of *observed* vs *predicted* values is separately reported for the fitting dataset (F, open symbols, n = 40) and the validation dataset (V, filled symbols, n = 22). In each scatterplot, Pearson's *r* and the associated *p*-value refer to the $y = x$ regression line (see main text for further details).



Supplementary Figure S4. Results of PCR modelling of *Pseudevernia furfuracea* element content in Italy for Cu, Fe, Ge, Hf, Hg, K, La, Li, Mg, Mn, Mo, Na. For each element, a scatterplot of *observed* vs *predicted* values is separately reported for the fitting dataset (F, open symbols, n = 40) and the validation dataset (V, filled symbols, n = 22). In each scatterplot, Pearson's *r* and the associated *p*-value refer to the $y = x$ regression line (see main text for further details).



Supplementary Figure S5. Results of PCR modelling of *Pseudevernia furfuracea* element content in Italy for Nb, Ni, P, Pb, Pd, Rb, S, Sb, Sc, Se, Sn, Sr. For each element, a scatterplot of *observed* vs *predicted* values is separately reported for the fitting dataset (F, open symbols, n = 40) and the validation dataset (V, filled symbols, n = 22). In each scatterplot, Pearson's *r* and the associated *p*-value refer to the $y = x$ regression line (see main text for further details).



Supplementary Figure S6. Results of PCR modelling of *Pseudevernia furfuracea* element content in Italy for Th, Ti, U, V, Y, Zn, Zr. For each element, a scatterplot of *observed* vs *predicted* values is separately reported for the fitting dataset (F, open symbols, n = 40) and the validation dataset (V, filled symbols, n = 22). In each scatterplot, Pearson's *r* and the associated *p*-value refer to the $y = x$ regression line (see main text for further details).

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Background element content in the lichen *Pseudevernia furfuracea*: A comparative analysis of digestion methods

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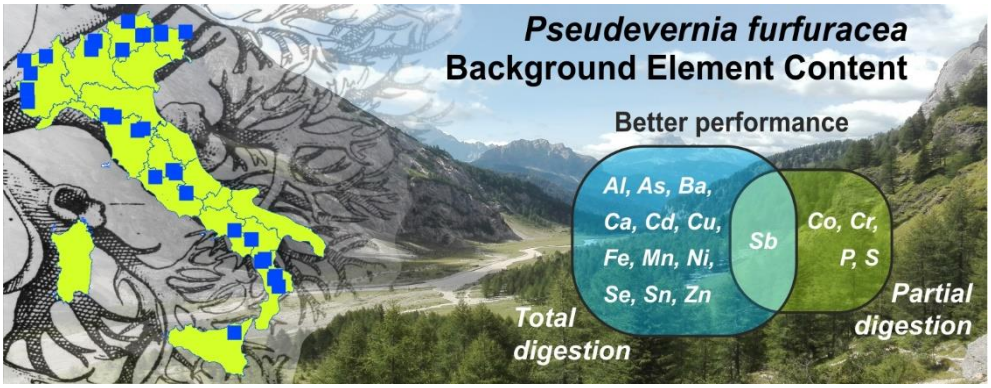
Highlights

• Acid digestion of samples is an integral part of multi-element quantification.
• Sample digestion methods affect the interpretation of bioaccumulation results.
• Background Element Content (BEC) values are needed to assess pollution levels.
• Digestions with and without HF were compared using *Pseudevernia furfuracea* samples.
• New HF-based BEC values are provided for this highly-performing biomonitor.

Abstract

In bioaccumulation studies, the interpretation of pollutant contents in the target biomonitor has to be performed by assessing a deviation from an unaltered reference condition. A common strategy consists in the comparison with Background Element Content (BEC) values, often built up by uncritically merging methodologically heterogeneous data. In this respect, the acid digestion of samples was identified as a major step affecting BEC data. Here, the analytical outcomes of two acid mixtures were compared on a set of matched paired samples of the lichen *Pseudevernia furfuracea*, a widely used biomonitor for which BEC values based on partial digestion were previously provided. The standard reference material BCR 482 (*P. furfuracea*) was used to validate analytical procedures consisting of either a HF total mineralization or an *aqua regia* partial one, both associated to ICP-MS multi-element analysis. In particular, the performance of the procedures was evaluated by comparing analytical results of field samples with the accuracy obtained on BCR aliquots (measured-to-expected percentage ratio). The total digestion showed a better performance for Al, As, Ba, Ca, Cd, Cu, Fe, Mn, Ni, Se, Sn and Zn, whereas the opposite was found for Cr, Co, P and S. Moreover, new BEC values were provided for *Pseudevernia furfuracea* using a consolidated statistical approach, after a total sample digestion with hydrofluoric acid. The multivariate investigation of the background variability of 43 elements in 57 remote Italian sites led to the identification of geographically homogeneous areas for which BEC values are provided for use as reference in biomonitoring applications.



Keywords: Air pollution; baseline; bioaccumulation; biomonitor; mineralization; acid extraction.

1. Introduction

Bioaccumulation of trace elements from atmospheric depositions is widely evaluated in lichens, symbiotic organisms with peculiar morphological and physiological traits, which allow their elemental content to reflect the chemical composition of the air (Bargagli 1998; Nash 2008). In bioaccumulation studies, the interpretation of the pollutant contents in the target biomonitor has to be performed by assessing a deviation from an unaltered reference condition. A common strategy consists in the comparison with Background Element Content (BEC) values, i.e. element concentration values measured in samples collected in remote areas, far distant from known emission sources (Bargagli 1998).

In a recent contribution (Cecconi et al. 2018), the review-based procedure used to assess lichen BEC values was extensively discussed, and several methodological criticalities were highlighted within so-obtained reference values. Such criticalities were further confirmed by an extensive survey of lichen biomonitoring literature spanning over 50 years of "established" practices. A remarkable variability of methods concerned almost all the steps of biomonitoring procedures, ranging from the processing of lichen material to the digestion and quality assurance procedures. Unexpectedly, details on acid digestion were often missing in the biomonitoring literature, possibly introducing a bias in BEC values built up on highly heterogeneous sample digests, as affecting accuracy for specific elements. The acid digestion is actually an integral part of most multi-element measurement procedures (Gaudino et al. 2007). Accordingly, the effects of different mineralization protocols were often addressed in the context of methodological studies of environmental chemistry concerning soil (e.g., Carvalho Vieira et al. 2005; Yafa and Farmer 2006; da Silva et al. 2014), sediments and sludges (e.g., Sastre et al. 2002) and biological matrices, from plant materials (Rodushkin et al. 1999; Tuncel et al. 2004; Rashid et al. 2016), lichens included (Baffi et al. 2002), to fish tissues (Ashoka et al. 2009).

Acid mixtures for wet sample digestion are traditionally subdivided in two main groups, according to the presence/absence of hydrofluoric acid (Gaudino et al. 2007). 'Total' digestions include hydrofluoric acid (HF), whereas 'partial' digestions (also referred as 'strong acid digestion' or '*aqua regia*') do not (Niazi et al. 1993; Cook et al. 1997; Perez-Santana et al. 2007). Furthermore, *aqua regia* mixtures are sometimes referred as 'acid leaching' procedures rather than 'acid digestions', since often they do not permit the determination of total analyte contents (Castilho et al. 2012). By contrast, it is generally agreed that total, HF-based digestions produce more accurate results for materials containing resistant mineral phases (e.g., Sandroni and Smith 2002), because they ensure a more performing dissolution of aluminosilicates (Yafa and Farmer 2006). Nevertheless, several drawbacks were also highlighted in relation to the use of HF when digesting airborne particulate matter samples. For instance, its use may determine the formation of volatile fluorides, eventually lost during sample preparation (Castilho et al. 2012), or insoluble calcium fluoride (CaF_2), which can co-precipitate metals, hence lowering element recoveries (Rönkkömäki et al. 2008). Most importantly, digestions with HF pose health concerns for operators (Le Fèvre and Pin 2005), have higher costs (Goddard and Brown 2014), and can damage the instrumentation (Silva et al. 2014). On these bases, alternative acid mixtures consisting of fluoroboric acid (HBF_4),

nitric acid (HNO_3) and *aqua regia* were also developed for the analysis of particulate matter collected by quartz filters (Castilho et al. 2012).

The selection of a proper digestion approach should rely on element speciation form, matrix character of the sample, and evaluation of possible interferences among elements (Kalembkiewicz and Sitarz-Palczak 2001). In the majority of cases, this does not fall within the bounds of practical feasibility for biomonitoring surveys, with their numerous, low-biomass sample batches, often analysed by external accredited laboratories. In these conditions, it is often not practically and economically viable to select different digestions techniques for the analysis of different sets of elements. As a matter of fact, the use of HF-based digestions to analyse lichen samples is becoming more common in the last years, as a painstaking analysis of lichenological literature revealed (Cecconi et al. 2018). However, an *aqua regia* partial digestion coupled with ICP-MS determination was used to define BEC values of the epiphytic lichen *Pseudevernia furfuracea*, one of the most widely used, highly performing biomonitor, on the basis of samples from 62 remote Italian sites (Cecconi et al. 2018). Baffi et al. (2002) obtained different results for *P. furfuracea* (in the form of reference material BCR 482; see Quevauviller et al. 1996), by using 'total' vs. 'partial' digestion mixtures. Since no solubilisation procedure meets the needs of all analytical techniques or solve all type of problems for trace element determination (Bettinelli et al. 2002), in this work new BEC values are proposed for this important biomonitor, based on a 'total' sample digestion instead of a 'partial' one, as a further contribution to the methodological standardization of lichen biomonitoring techniques (Incerti et al. 2017; Cecconi et al. 2018, 2019) aimed at enhancing proper data comparison.

2. Materials and methods

2.1 The target species

Pseudevernia furfuracea is a fruticose, meso-xerophilous and photophilous lichen, occurring on acidic, non-eutrophicated bark, with optimum in cool-temperate/boreal areas (Rikkinen 1997; Smith et al. 2009). *P. furfuracea* has two morphologically undistinguishable varieties (var. *furfuracea* and var. *ceratea*), with richly branched thalli often densely covered by finger-shaped outgrowths (isidia), which considerably increase particle entrapment (Riga-Karandinos and Karandinos 1998; Bargagli and Mikhailova 2002). The large thallus size and the easy identification in the field ensure fast and easy sampling and preparation. In addition, the availability of very flourishing and abundant populations definitely limits the risk of population impoverishment by dedicated sampling campaigns. For these reasons, *P. furfuracea* is frequently used in biomonitoring surveys of trace elements (e.g., Tretiach et al. 2011a; Gallo et al. 2017), it has already been used for the production of a standard reference material (Quevauviller et al. 1996) and targeted for the application-driven determination of BEC values (Cecconi et al. 2018).

2.2 Lichen sampling and processing

Thalli of *Pseudevernia furfuracea* without distinction of the two varieties (Incerti et al. 2017) were collected at 57 remote sites of the main Italian mountain ranges (Supplementary Table S1). The

source lichen material was largely the same used in a previous study (Cecconi et al. 2018), with the addition of lichen thalli sampled in previously unexplored areas (Supplementary Table S1).

Details on lichen sampling are provided in Incerti et al. (2017). In the laboratory, the lichen material was dried out at room temperature until reaching constant mass. All thalli were carefully cleaned from fragments of tree bark, and other lichens and mosses using powder free gloves and plastic tweezers. Terminal lobes homogenous in size (15–25 mm), shape, and isidia density (visually assessed) were selected, separated from the thallus using ceramic scissors, and pulverized through a planetary ball mill (Retsch PM100), with milling cycles of 4 min at 550 rpm. Once processed, the lichen material was sealed under vacuum and stored at -20°C until analytical determination. Overall, 171 analytical replicates (3 replicates × 57 sites) underwent a total digestion; in 20 cases out of 57, the number of replicates was doubled in order to perform also a partial sample digestion (additional 3 replicates × 20 sites; Supplementary Table S1).

2.3 Analytical procedures

All analytical replicates were submitted to multielement analysis at Bureau Veritas Mineral Laboratories (BVML, former ACME Analytical Laboratories, Vancouver, Canada), a widely acknowledged high-quality data provider for element content in different matrices, including lichens (e.g., Tretiach et al. 2011b; García-Ordiales et al. 2016). The element concentrations were determined for all the samples after a multi-acid digestion with perchloric and hydrofluoric acid. In addition, the coupled set of analytical replicates (60 replicates from 20 sites) were subjected to a HF-free, partial digestion. Regarding the total digestion, samples were analysed at BVML according to the MA250 analytical packet (protocol for Ultra Trace ICP-MS chemical analysis of multi-acid digested samples). Replicate aliquots of 0.25 g were heated in HNO_3-$HClO_4$-HF solution (volume ratio 1:1:2) until fuming and then dried; the resulting residue was dissolved in 50% HCl solution and heated using a mixing hot block. After cooling the solutions were transferred to test-tubes and brought to volume using HCl. The use of strong oxidizing acids (HNO_3 and $HClO_4$) ensures the removal of organic matter, while the use of HF enables the dissolution of silicates, allowing the near-total dissolution of the mineral fraction (Yafa and Farmer 2006). Regarding the partial digestion, samples were analysed according to the VG101-EXT analytical packet (protocol for dry plant material analysis). Replicates aliquots of 1.0 g were cold leached with HNO_3 for 1 h, then digested in a hot water bath. After cooling, an *aqua regia* solution (HCl-HNO_3, volume ratio 3:1) was added to each sample to leach in a heating block of boiling water bath. Samples were made up to volume with dilute HCl and then filtered.

The content of 59 (MA250 packet) and 53 elements (VG101-EXT packet) was analysed through ICP-MS with a Perkin Elmer Elan 6000 ICP mass spectrometer. All the reagents used were at least ACS-grade. Values were expressed on a dry mass basis (μg g^{-1}).

BVML quality assurance/control (QA/QC) protocol includes a sample-prep blank carried through all stages of preparation and analysis as the first sample, a pulp duplicate to monitor analytical precision, two reagent blanks to measure background, and aliquots of in-house Standard Reference Materials. In-house standards V16 and CDV-1 (plant leaves) were used to monitor accuracy in the case of partial digestion protocol on 38 and 44 elements, respectively, each with 11 replicates. OREAS25A-4A and OREAS45E (soil) were used in the case of total digestion on 39 and

57 elements, respectively, each with 5 replicates. The standard reference material CRM 482 'lichen' *P. furfuracea* was also sent to BVML to be blindly analysed with both packets. Quality control was expressed in terms of mean recovery percentages, calculated as the percentage ratio between the measured and the expected values for the lichen standard BCR 482 and for the internal standards (Table 1, Supplementary Table S2). For BCR 482, mean recoveries were calculated for elements with either certified or indicative expected values (Quevauviller et al. 1996). Accuracy was deemed satisfactory when mean recovery percentage was between 80 and 120% of the expected value.

Table 1 Element content (µg g^{-1}) of the epiphytic lichen *Pseudevernia furfuracea* as resulted from the chemical analyses of 20 samples either subjected to total or partial digestion (MA250 and VG101-EXT packets, respectively). Data refer to mean ± standard deviation and instrumental lower Limit of Detection (LoD) for the 47 elements analysed with both analytical packets. Mean recovery percentages and 95% confidence interval (95% C.I.) are reported for the standard reference material BCR 482 'lichen' *P. furfuracea*; mean recoveries and confidence interval were calculated on 5 analytical replicates. Results of statistical testing for differences between element content of samples subjected to total (T) and partial (P) digestion are reported (Wilcoxon matched pair test, significant p-values in italic). The ratio between mean element content values and mean recovery percentages from total (T) and partial (P) digestion are also reported (T/P and T/P recovery, respectively). Wilcoxon test results and T/P ratios were not reported for elements with null variance.

Element	Total digestion (MA250)			Partial digestion (VG101-EXT)			Wilcoxon		T/P	T/P recovery
	Mean ± SD	LoD	BCR 482 recovery (95% C.I.)	Mean ± SD	LoD	BCR 482 recovery (95% C.I.)	z	*p*-value		
Ag*	0.033 ± 0.008	0.02	-	0.021 ± 0.008	0.002	-	6.57	*≤ 10^{-6}*	1.57	-
Al•	1038 ± 387	200	99.7 (99.7 ÷ 99.7)	455 ± 166	100	36.3• (36.3 ÷ 36.3)	6.74	*≤ 10^{-6}*	2.28	2.75
As	0.555 ± 0.346	0.2	84.7 (40.4 ÷ 129.0)	0.333 ± 0.222	0.1	80.0 (73.5 ÷ 86.5)	6.74	*≤ 10^{-6}*	1.67	1.06
Ba*•	16.2 ± 6.6	1	95.3 (91.6 ÷ 99.0)	12.2 ± 5.7	0.1	65.4• (63.7 ÷ 67.0)	6.74	*≤ 10^{-6}*	1.33	1.46
Be*§	1.0 ± 0.0	1	-	0.1 ± 0.0	0.1	-				-
Bi	0.056 ± 0.026	0.04	-	0.047 ± 0.027	0.02	-	4.25	*2.1 · 10^{-5}*	1.19	-
Ca	7467 ± 3410	100	102.5 (101.5 ÷ 103.6)	7237 ± 3221	100	86.1 (81.9 ÷ 90.4)	3.52	*4.3 · 10^{-4}*	1.03	1.19
Cd	0.203 ± 0.066	0.02	95.0 (91.0 ÷ 99.0)	0.160 ± 0.059	0.01	94.3 (91.9 ÷ 96.7)	6.57	*≤ 10^{-6}*	1.27	1.01
Ce	1.61 ± 0.74	0.02	-	1.08 ± 0.51	0.01	-	6.74	*≤ 10^{-6}*	1.49	-
Co*	0.287 ± 0.085	0.2	115.6 (98.3 ÷ 133.0)	0.268 ± 0.077	0.01	88.8 (86.6 ÷ 90.9)	2.42	*0.016*	1.07	1.30
Cr*•	5.90 ± 1.13	1	150.5• (125.3 ÷ 175.7)	2.98 ± 0.53	0.1	101.5 (97.0 ÷ 105.9)	6.74	*≤ 10^{-6}*	1.98	1.53
Cs*	0.175 ± 0.111	0.1	-	0.132 ± 0.108	0.005	-	6.65	*≤ 10^{-6}*	1.33	-
Cu*	5.68 ± 2.22	0.1	97.9 (94.4 ÷ 101.4)	5.56 ± 2.1	0.01	94.0 (90.4 ÷ 97.6)	2.78	*0.005*	1.02	0.99
Fe*	618 ± 216	100	97.5 (94.7 ÷ 100.3)	557 ± 198	10	90.3 (86.8 ÷ 93.8)	5.99	*≤ 10^{-6}*	1.11	0.97
Ga*	0.277 ± 0.11	0.02	-	0.127 ± 0.045	0.1	-	6.66	*≤ 10^{-6}*	2.18	-
Hf*	1.356 ± 0.569	0.02	-	0.064 ± 0.035	0.001	-	6.74	*≤ 10^{-6}*	21.19	-
In§	0.01 ± 0.00	0.01	-	0.02 ± 0.00	0.02	-				-
K	3470 ± 617	100	96.4 (94.7 ÷ 98.2)	3057 ± 593	100	103.6 (100.0 ÷ 107.2)	6.27	*≤ 10^{-6}*	1.14	0.93
La*	0.818 ± 0.407	0.1	-	0.496 ± 0.247	0.01	-	6.74	*≤ 10^{-6}*	1.65	-
Li*	0.642 ± 0.286	0.1	-	0.369 ± 0.139	0.01	-	6.49	*≤ 10^{-6}*	1.74	-
Mg*	832 ± 148	100	-	805 ± 143	10	-	3.01	*0.003*	1.03	-
Mn	61.2 ± 27.2	1	93.3 (90.2 ÷ 96.5)	58.0 ± 27.1	1	92.1 (87.8 ÷ 96.4)	4.82	*≤ 10^{-6}*	1.06	1.01
Mo•	0.306 ± 0.207	0.05	42.8• (39.5 ÷ 46.2)	0.307 ± 0.209	0.01	44.7• (38.6 ÷ 50.8)	0.16	0.870	1.00	0.85
Na	142.0 ± 84.6	10	-	84.3 ± 85.4	10	-	6.74	*≤ 10^{-6}*	1.68	-
Nb	0.175 ± 0.067	0.04	-	0.046 ± 0.019	0.01	-	6.74	*≤ 10^{-6}*	3.80	-
Ni	1.96 ± 1.01	0.1	102.0 (95.5 ÷ 109.0)	1.57 ± 0.87	0.1	89.1 (84.0 ÷ 94.1)	6.55	*≤ 10^{-6}*	1.25	1.27
P	558 ± 194	10	89.0 (86.9 ÷ 91.0)	580 ± 188	10	98.0 (93.1 ÷ 102.8)	3.73	*1.9 · 10^{-4}*	0.96	0.91
Pb	3.87 ± 1.98	0.02	89.6 (87.3 ÷ 91.8)	3.49 ± 1.83	0.01	89.7 (86.3 ÷ 93.0)	6.62	*≤ 10^{-6}*	1.11	1.00
Rb	12.26 ± 5.13	0.1	-	10.91 ± 4.7	0.1	-	6.74	*≤ 10^{-6}*	1.12	-
Re§	0.002 ± 0.000	0.002	-	0.001 ± 0.000	0.001	-				-
S•	1073 ± 288	400	71.8• (69.1 ÷ 74.5)	1178 ± 308	100	96.0 (85.0 ÷ 107.1)	3.61	*3.1 · 10^{-4}*	0.91	0.75
Sb	0.123 ± 0.075	0.02	88.0 (72.3 ÷ 103.7)	0.125 ± 0.074	0.02	83.4 (77.1 ÷ 89.8)	1.17	0.240	0.98	1.06
Sc	0.182 ± 0.093	0.1	-	0.340 ± 0.069	0.1	-	6.18	*≤ 10^{-6}*	0.54	-
Se•	0.507 ± 0.189	0.3	104.0 (94.9 ÷ 114.0)	0.357 ± 0.145	0.1	70.0• (60.7 ÷ 79.3)	4.98	*≤ 10^{-6}*	1.42	1.52
Sn•	0.413 ± 0.227	0.1	106.9 (89.1 ÷ 124.6)	0.411 ± 0.231	0.02	156.8• (92.3 ÷ 221.3)	0.35	0.723	1.00	0.68

Table 1 (continued)

Element	Total digestion (MA250)			Partial digestion (VG101-EXT)			Wilcoxon		T/P	T/P recovery
	Mean ± SD	LoD	BCR 482 recovery (95% C.I.)	Mean ± SD	LoD	BCR 482 recovery (95% C.I.)	z	*p*-value		
Sr	16.50 ± 7.52	1	-	14.62 ± 6.10	0.5	-	6.07	$\leq 10^{-6}$	1.13	-
Ta*§	0.100 ± 0.000	0.1	-	0.002 ± 0.001	0.001	-				-
Te	0.094 ± 0.046	0.05	-	0.020 ± 0.001	0.02	-	6.74	$\leq 10^{-6}$	4.70	-
Th*	0.167 ± 0.073	0.1	-	0.104 ± 0.039	0.01	-	6.57	$\leq 10^{-6}$	1.61	-
Ti*	66.17 ± 26.43	10	-	11.68 ± 3.36	1	-	6.74	$\leq 10^{-6}$	5.67	-
Tl§	0.052 ± 0.006	0.05	-	0.020 ± 0.000	0.02	-				-
U*§	0.100 ± 0.000	0.1	-	0.024 ± 0.013	0.01	-				-
V	1.90 ± 0.77	1	85.6 (70.7 ÷ 100.4)	2.35 ± 0.48	2	101.6 (86.8 ÷ 116.5)	4.23	$2.4 \cdot 10^{-5}$	0.81	0.84
W	0.118 ± 0.047	0.1	-	0.117 ± 0.046	0.1	-	0.42	0.673	1.00	-
Y*	7.068 ± 4.09	0.1	-	0.636 ± 0.281	0.001	-	6.74	$\leq 10^{-6}$	11.11	-
Zn	43.4 ± 20.9	0.2	96.7 (94.3 ÷ 99.1)	39.6 ± 19.3	0.1	90.1 (88.6 ÷ 91.6)	6.18	$\leq 10^{-6}$	1.10	1.03
Zr*	58.51 ± 24.5	0.2	-	2.83 ± 1.36	0.01	-	6.74	$\leq 10^{-6}$	20.67	-

2.4 Data analysis

The median values of the 47 elements analysed by ICP-MS after partial and total digestion methods were tested for significant differences using Wilcoxon's matched pair test, considering all 60 analytical replicates for each digestion method. In order to assess which digestion method had the better performance, a comparative analysis was conducted taking into account the results of analytical determination on both field samples and the standard BCR 482 replicates blindly included in the batch, following the flow illustrated in Fig.1.

The pipeline followed to obtain the BECs for totally-digested samples of *Pseudevernia furfuracea* at the national level is the same used in Cecconi et al. (2018). First, extreme outliers (OLs) were removed for each element, as possibly affected by unexpected anthropogenic contributions at the sampling sites. Extreme OLs were identified according to the Tukey's method (i.e. values higher than the 3rd quartile of the distribution plus 3 times the interquartile range), which makes no distributional assumptions, thus being applicable to skewed or non-bell-shaped data distributions (Hoaglin et al. 1986). Then, the elements with at least 50% of field values below the lower limit of detection (LoD) were excluded from further data analysis. Finally, the content data matrix of 57 collection sites × 43 elements, with data standardized for each element to account for different content magnitude in different elements, was submitted to Cluster Analysis (CA, with Euclidean distance as distance measure and Ward's method as grouping algorithm) and Principal Component Analysis (PCA). Both CA and PCA were separately performed for elements and collection sites. For the resulting clusters of sites, the mean standardized content of each group of elements was calculated, and a complete series of basic descriptive statistics of element content was separately provided for each element, after the outlier removal. In detail, mean, standard deviation, median, median absolute deviation (MAD; Reimann et al. 2005), and 98th percentile, were calculated for each element.

All data analyses and graphics were performed with the software packages Statistica v. 10 (StatSoft Inc., Tulsa, OK) and R (R Core Team 2013). Statistical significance was tested at $\alpha = 0.05$ in all cases.

3. Results and Discussion

3.1 Accuracy of the analytical procedures

When compared to certified or indicative values (Quevauviller et al. 1996; Table 1), mean recoveries in the lichen standard BCR 482 were either below 80% or above 120% limited to 3 (i.e., Cr, Mo and S) and 5 (Al, Ba, Mo, Se, Sn) elements out of 21, for totally- and partially-digested replicates, respectively (Table 1). Total and partial recovery medians in BCR 482, as tested for significant differences using Wilcoxon's matched pair test, were significantly higher ($p < 0.05$) in partially-digested replicates for K, P, and S, while Al, Ba, Ca, Co, Fe, Ni, Se, and Zn showed the opposite trend.

For the total digestion, recoveries obtained on the in-house standard OREAS25A-4A were always included between 80% and 120% for all the 39 tested elements, while those obtained on the standard OREAS45E were either below 80% or above 120% limited to 4 cases out of 55 (Cd, Tb, Te and Tl). When considering the partial digestion, recoveries obtained on the standard CDV-1 and V16 exceeded the 80-120% range in 8 cases out of 44: Ge, S, Se, V (CDV-1) and Au, Ga, La, S (V16) (Supplementary Table S2).

3.2 Comparison of analytical results after total and partial digestion of samples

Overall, the elemental content of totally-digested samples was higher than that of matched partially-digested ones. Indeed, in 33 cases out of the 41 tested elements (80.5%), totally-digested samples had significantly higher median content values, whereas the opposite situation was highlighted for P, S, Sc, and V. For Mo, Sb, Sn and W the two digestions did not produce significantly different results. Among the 33 elements showing significantly higher values in totally-digested samples, Al, Ga, Hf, Nb, Te, Ti, Y and Zr showed the most pronounced differences (Total/Partial ratio, Table 1), with mean element content values of totally-digested samples ranging from c. 2 times (Al, Ga) to c. 20 times (Hf, Zr) the value of matched partially-digested samples. For the elements showing the opposite trend, the differences were far less pronounced, with mean values of totally-digested samples ranging from 0.54 times (Sc) to 0.96 times (P) the value of matched samples (Table 1).

Following the flow illustrated in Fig.1 and considering the results of analytical determination on field samples and BCR 482 (Table 1), it was possible to evaluate the relative performance of the two digestion methods. In particular, the HF digestion showed a better performance for Al, As, Ba, Ca, Cd, Cu, Fe, Mn, Ni, Se, Sn and Zn; partial digestion showed a better performance limited to the lichen macronutrients P and S, and for Cr and Co. The two digestions methods showed comparable performance for Sb, whereas for Mo both procedures produced comparable but very low recoveries, hence unreliable. It was not possible to draw conclusive results for V (due to the differences in the LoDs associated to the two analytical packets and to the generally low median values characterizing both sets; Table 1, Fig. 1) as well as for all the elements with missing certified or indicative values.

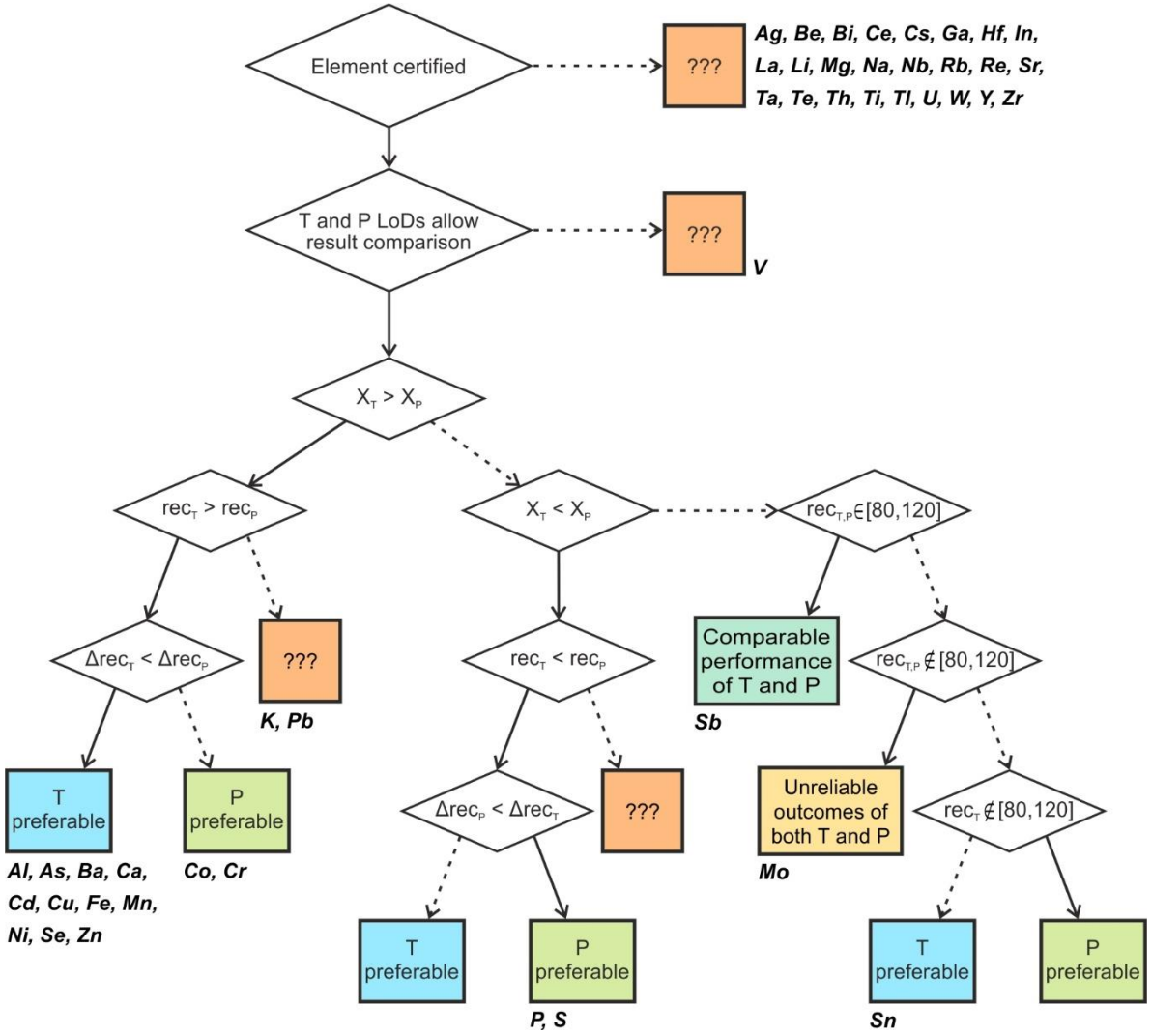


Fig. 1 Flow chart illustrating the procedure used to assess the relative performance of total (T) and partial (P) digestion methods. In the flow chart, full and dotted arrows indicate negative and positive statements, respectively; X_T and X_P refer to the median values of a generic chemical element X, similarly, rec_T and rec_P indicate the recovery percentages obtained after total and partial digestion of samples. Δrec_T and Δrec_P indicate the offset from the 100% recovery ($\Delta rec_T = |100 - rec_T|$, $\Delta rec_P = |100 - rec_P|$). Boxes with question marks indicate the impossibility to draw conclusive results due to (*i*) the lack of certified values, (*ii*) differences in LoDs having repercussions on results comparison and (*iii*) inconsistent results between element content values and recoveries.

3.3 Evaluation of the performance of total and partial digestion methods

The acid attack is an integral part of the analytical measurement, and it is widely acknowledged as a factor influencing the elemental content results (Gaudino et al. 2007). Indeed, the effects of the use of different acid mixtures for the mineralization of environmental and biological samples, were frequently investigated over the last twenty years (e.g., Chen and Ma 1998; Rodushkin et al. 1998; Baffi et al. 2002; Sastre et al. 2002; Ashoka et al. 2009).

A total digestion scheme must include the use of hydrofluoric acid (HF) to completely release the trace elements included in the aluminosilicate phase (Sastre et al. 2002), however, the use of HF consistently enlarges the timing and costs of the procedure, requiring the adoption of safety measures and highly trained personnel (Le Fèvre and Pin 2005). Nonetheless, HF-free digestions, although generally safe and cheap, may lead to the underestimation of the content of environmental-concerning trace elements held at aluminosilicate sites (Tam and Yao 1999).

In order to reliably compare our results on the relative performance of digestion methods to those obtained in the context of different studies on the same lichen matrix, the use of a comparable technique for multi-element determination should be considered as a fundamental constraint. In this study, the results on field samples and certified material aliquots were analysed through ICP-MS, an analytical technique used also by Baffi et al. (2002). These authors showed that the digestion in microwave oven with HF-HNO_3-H_2O_2 was more suitable than the digestion with HNO_3-H_2O_2 for Al, Ba, Cr, Cu, Fe, Mn, V, and Zn. Our results are in substantial agreement with such findings: in particular, the total digestion better performed for Al, Ba, Cu, Fe, Mn and Zn. On the other hand, the content of Cr in totally-digested samples was importantly overestimated (150%, Table 1), apparently making the HF-free digestion preferable for the quantification of this element. Interestingly, Rönkkömäki et al. (2008) pointed out that the formation of low solubility chromyl fluoride (CrO_2F_2) may compromise Cr recovery. By contrast, several authors besides Baffi et al. (2002) reported better recoveries with total digestions for Cr (e.g., Kackstaetter and Heinrichs 1997; Chen and Ma 2001), although the same authors noticed the occurrence of spectral interference during the ICP-MS determination of ^{52}Cr, which possibly affected the results. As a matter of fact, ICP-MS techniques may suffer from interferences caused by polyatomic ions formed from precursors having different sources (such as the sample matrix, reagents used for plasma gases preparation, and entrained atmospheric gases; May and Wiedmeyer 1998).

Limited to V, our results cannot be considered fully conclusive since the LoDs obtained by the analytical packets VG101-EXT and MA250 were limiting for the interpretation of the results (Table 1). Indeed, the LoDs obtained with total and partial digestion packets were 1 and 2 μg g^{-1}, respectively (Table 1), and the overall number of records below or equal to total and partial LoDs were 35% and 65%, therefore affecting median values as well as the outcome of statistical testing. As far as K and Pb, the median values obtained by total digestion were higher than those obtained by partial digestion, however, the opposite situation was found for the recovery percentages on the certified lichen replicates (Fig. 1, Table 1), suggesting that the source of measurement bias could be unrelated to the acid attack. Nonetheless, it should also be considered that the lichen CRM is generally characterized by a low content of terrigenous particulate and may not always give fully consistent indications about the degree of mineralization obtained on real samples (Bettinelli et al. 2002) with respect to some elements mainly held at aluminosilicate sites such as Pb (Tam and Yao 1999).

Interestingly, for the lichen macronutrients P and S, as well as for Co (Nash 2008), the partial digestion provided the best accuracy. Limited to P and S, our results were in contrast with those of Chen and Ma (1998) on soil samples. However, the relative abundance of organic and inorganic P and S is acknowledged as a source of variability for analytical results (Sastre et al. 2002; Hamilton et al. 2015), along with the major interferences affecting the most abundant isotopes of P and S, possibly resulting from nitrogen, oxygen and hydrogen (and their molecular ions: e.g., $^{14}N^{16}O^1H^+$, $^{15}N^{16}O^+$, $^{14}N^{17}O^+$, $^{13}C^{18}O^+$, $^{12}C^{18}O^1H^+$; May and Wiedmeyer 1998) during ICP-MS determination (Bandura et al. 2002). Furthermore, it must be considered that volatilization during fuming may result in some loss of S during the total MA250 digestion (BVML technical information package; Castilho et al. 2012), which could potentially clarify the observed pattern for this element.

3.4 Geographical pattern of background element content

The cluster analysis based on the element content data from the field sampling produced four main clusters of sites (Fig. 2A, Fig. 3) separated for geographical location. Cluster I included 14 sites located in the eastern Alps (with the exception of site 8, located in the western Alps, and site 41, located in the northern Apennines), generally characterized by sedimentary carbonate substrates; cluster II included 11 sites, located in the western Alps, exclusively over metamorphic siliceous rocks; cluster III included 13 sites located in the central Alps, mostly characterized by metamorphic substrates. Finally, cluster IV included 19 sites located in the Apennines (with the exception of site 5 located in the western Alps) over heterogeneous lithological substrates. When removing from clusters the few sites inconsistent for geographic location (i.e., sites 8 and 41 from cluster I and site 5 from cluster IV), four geographically homogeneous sets of lichen samples were obtained (Supplementary Fig. S1). A clear pattern of lichen element composition emerged from the PCA (Fig. 2B): in particular, elements of group 1 (Ag, As, Bi, Cs, Cu, Mo, Rb, S, Sb, Se, Sn, Zn) were consistently placed at high scores of the second principal component (PC II), inversely related to longitude and positively to latitude. Differently, elements of group 2 (Al, Cd, Ce, Fe, Ga, Gd, La, Li, Na, Nb, Pb, Sc, Sm, Th, Ti, V), including several elements of lithogenic origin, were placed at negative scores of the first axis (PC I), positively related to longitude and inversely to latitude. Elements of group 3 (Ba, Ca, Co, Cr, Hf, K, Mg, Mn, Nd, Ni, P, Sr, Te, Y, Zr) were placed at negative scores of PC I, hence negatively related to latitude, but scattered along PC II.

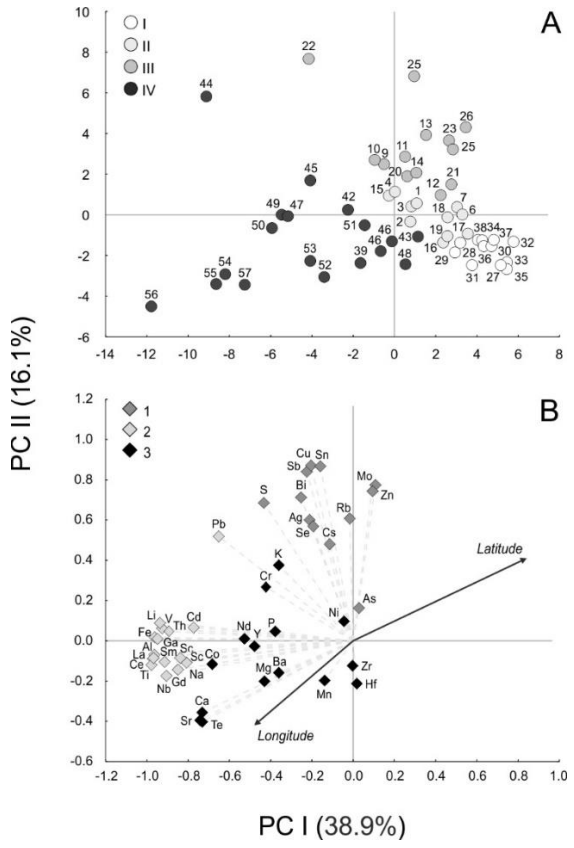


Fig. 2 Results of multivariate analysis of element content in *Pseudevernia furfuracea* samples collected at 57 remote field sites in Italy. (A) Factorial scores of field sites, symbolized according to CA results of Fig. 3. (B) PCA plot showing loading vectors of the elements symbolized according to the Cluster Analysis (CA) and their relationship with geographical location of the field sites (i.e. longitude and latitude), plotted as supplementary variables (Legendre and Legendre 1998).

Such geographical correspondence is better noticeable in the plot of collection sites in the ordination space (Fig. 2A). Indeed, the sampling sites were distributed in the ordination space according to element content gradients. In particular, Apennine sites of cluster IV were placed at negative scores of PC I, differently from the clusters of Alpine sites (i.e., clusters I-III), which were instead placed at positive scores of PC I. The latter clusters slightly segregated along PC II. Indeed, these were characterized by increasing averaged scores along this axis (-1.64, -0.17, and 3.32, for sites of cluster I, II and III, respectively). Consistently, the clusters of sites at different geographic locations showed significant differences of standardized content of the three groups of elements (Fig. 3). Eastern Alpine sites of cluster I showed the lowest standardized element content of all the three element groups. Namely, cluster I had the lowest content of 26 elements out of 43 (Table 2). Western Alpine sites of cluster II showed negative standardized content of element groups 1 and 2 and positive content of element group 3; differently, central Alpine sites of cluster III showed the highest levels of elements of group 1, but negative element content of groups 2 and 3. In particular, cluster II exhibited lower elemental content than cluster III for 19 elements (either significantly or not), including As, Bi, Cu, Rb, S, Sb, Se, Sn, Zn (group 1) and Pb (group 2); the opposite situation was highlighted limited to 5 elements, including Co, Cr and Ni (group 3) (Table 2). Finally, Apennine sites of cluster IV showed negative values of elements of group 1, but the highest standardized content of elements of group 2 and 3 (Fig. 3), also exhibiting the highest content of 22 out of 43 elements (Table 2).

Table 2 Background element content (BEC) values (μg g^{-1}) in the epiphytic lichen *Pseudevernia furfuracea* in Italy. BECs are reported for sites with similar lichen element content (site clusters according to CA results; Fig. 2A, Fig. 3). Data refer to mean ± standard deviation, median, median absolute deviation (MAD) and 98th percentile for 43 elements listed according to their group (according to CA results; Fig. 2B, Fig. 3). Between-groups differences (Kruskal-Wallis ANOVA; K-W, and significant *p*-values in italic) are reported for each element. Different letters above the median values indicate significantly different groups within each row (Dunn's post hoc test at $p < 0.05$).

Element (group)	Cluster I (Eastern Alps, n = 12)			Cluster II (Western Alps, n = 11)			Cluster III (Central Alps, n = 13)			Cluster IV (Apennines, n = 18)			K-W *p*-value
	Mean ± SD	Median (MAD)	98th %ile	Mean ± SD	Median (MAD)	98th %ile	Mean ± SD	Median (MAD)	98th %ile	Mean ± SD	Median (MAD)	98th %ile	
Ag (1)	0.030 ± 0.009	0.027^{a} (0.004)	0.052	0.030 ± 0.004	0.032^{ab} (0.003)	0.035	0.037 ± 0.010	0.034^{b} (0.002)	0.057	0.032 ± 0.008	0.030^{ab} (0.004)	0.049	0.0724
As (1)	0.611 ± 0.220	0.533^{b} (0.117)	1.000	0.398 ± 0.131	0.433^{a} (0.083)	0.567	0.605 ± 0.252	0.633^{b} (0.200)	1.000	0.407 ± 0.21	0.367^{a} (0.100)	0.967	*0.0083*
Bi (1)	0.046 ± 0.013	0.040^{a} (0.000)	0.090	0.046 ± 0.008	0.040^{a} (0.000)	0.060	0.066 ± 0.026	0.060^{b} (0.017)	0.137	0.053 ± 0.025	0.040^{ab} (0.000)	0.123	*0.0118*
Cs (1)	0.120 ± 0.041	0.100^{a} (0.000)	0.200	0.163 ± 0.165	0.100^{ab} (0.000)	0.600	0.286 ± 0.241	0.200^{b} (0.100)	0.800	0.154 ± 0.096	0.100^{a} (0.000)	0.500	*0.0085*
Cu (1)	4.50 ± 1.28	4.18^{a} (0.47)	8.37	4.79 ± 0.85	4.50^{a} (0.37)	6.00	7.36 ± 1.92	6.72^{b} (1.00)	12.70	4.95 ± 1.66	4.53^{a} (0.53)	10.97	*< 10-4*
Mo (1)	0.223 ± 0.193	0.160^{ab} (0.035)	0.828	0.340 ± 0.188	0.273^{bc} (0.043)	0.787	0.423 ± 0.139	0.373^{c} (0.090)	0.710	0.141 ± 0.058	0.140^{a} (0.037)	0.277	*< 10-4*
Rb (1)	7.36 ± 4.65	6.57^{a} (1.35)	18.77	10.11 ± 3.00	11.70^{a} (2.20)	13.90	19.97 ± 9.04	17.92^{b} (4.73)	43.00	10.49 ± 5.57	7.90^{a} (3.13)	21.00	*0.0001*
S (1)	797 ± 190	800^{a} (117)	1283	972 ± 170	1000^{ab} (100)	1167	1176 ± 256	1200^{c} (200)	1767	1077 ± 216	1000^{bc} (133)	1600	*0.0003*
Sb (1)	0.070 ± 0.039	0.057^{a} (0.020)	0.147	0.094 ± 0.029	0.097^{a} (0.020)	0.153	0.163 ± 0.066	0.140^{b} (0.015)	0.343	0.088 ± 0.039	0.077^{a} (0.017)	0.207	*< 10-4*
Se (1)	0.408 ± 0.068	0.400^{a} (0.050)	0.533	0.431 ± 0.054	0.433^{a} (0.033)	0.500	0.586 ± 0.134	0.583^{b} (0.133)	0.767	0.478 ± 0.158	0.467^{a} (0.117)	0.867	*0.0055*
Sn (1)	0.276 ± 0.110	0.267^{a} (0.067)	0.500	0.376 ± 0.167	0.333^{a} (0.100)	0.750	0.540 ± 0.213	0.517^{b} (0.117)	1.100	0.311 ± 0.153	0.267^{a} (0.067)	0.833	*0.0002*
Zn (1)	38.0 ± 12.2	38.7^{a} (8.2)	65.0	40.0 ± 9.5	37.1^{a} (2.3)	62.7	64.4 ± 18.3	61.8^{b} (12.1)	101.2	31.4 ± 10.3	32.2^{a} (8.6)	52.2	*< 10-4*

Table 2 (continued)

Element (group)	Cluster I (Eastern Alps, n = 12)			Cluster II (Western Alps, n = 11)			Cluster III (Central Alps, n = 13)			Cluster IV (Apennines, n = 18)			K-W *p*-value
	Mean ± SD	Median (MAD)	98th %ile	Mean ± SD	Median (MAD)	98th %ile	Mean ± SD	Median (MAD)	98th %ile	Mean ± SD	Median (MAD)	98th %ile	
Al (2)	688 ± 279	600[a] (100)	1333	835 ± 190	800[ab] (200)	1100	1024 ± 402	983[b] (217)	2067	1600 ± 485	1633[c] (433)	2533	*< 10-4*
Cd (2)	0.159 ± 0.037	0.153[a] (0.015)	0.235	0.155 ± 0.032	0.153[a] (0.030)	0.192	0.204 ± 0.059	0.200[ab] (0.023)	0.367	0.251 ± 0.098	0.227[b] (0.042)	0.537	*0.0003*
Ce (2)	1.07 ± 0.49	0.92[a] (0.18)	2.04	1.10 ± 0.28	1.20[a] (0.20)	1.47	1.47 ± 0.58	1.34[a] (0.30)	2.91	2.70 ± 0.91	2.79[b] (0.87)	4.19	*< 10-4*
Fe (2)	417 ± 173	400[a] (100)	800	537 ± 146	500[ab] (100)	800	612 ± 213	600[b] (117)	1067	868 ± 247	900[c] (200)	1300	*< 10-4*
Ga (2)	0.188 ± 0.067	0.183[a] (0.041)	0.350	0.221 ± 0.047	0.230[ab] (0.042)	0.280	0.286 ± 0.111	0.258[b] (0.065)	0.523	0.398 ± 0.119	0.387[c] (0.067)	0.637	*< 10-4*
Gd (2)	0.113 ± 0.025	0.100[a] (0.000)	0.183	0.104 ± 0.011	0.100[a] (0.000)	0.133	0.126 ± 0.030	0.133[a] (0.033)	0.200	0.189 ± 0.069	0.167[b] (0.067)	0.300	*0.0003*
La (2)	0.520 ± 0.249	0.433[a] (0.133)	0.967	0.557 ± 0.158	0.600[a] (0.117)	0.833	0.733 ± 0.31	0.667[a] (0.133)	1.433	1.407 ± 0.484	1.467[b] (0.467)	2.200	*< 10-4*
Li (2)	0.383 ± 0.230	0.300[a] (0.100)	0.900	0.463 ± 0.143	0.433[ab] (0.100)	0.733	0.655 ± 0.276	0.633[b] (0.117)	1.433	0.944 ± 0.27	0.967[c] (0.233)	1.400	*< 10-4*
Na (2)	82.4 ± 32.7	70.0[a] (6.7)	155.0	86.1 ± 20.4	80.0[a] (13.3)	126.7	108.8 ± 40.6	101.7[a] (21.7)	203.3	227.6 ± 54	233.3[b] (20.0)	323.3	*< 10-4*
Nb (2)	0.122 ± 0.042	0.127[a] (0.040)	0.198	0.155 ± 0.035	0.150[a] (0.027)	0.223	0.159 ± 0.054	0.158[a] (0.042)	0.270	0.286 ± 0.093	0.287[b] (0.083)	0.463	*< 10-4*
Pb (2)	2.77 ± 0.98	2.71[a] (0.44)	4.79	2.35 ± 0.57	2.38[a] (0.19)	3.15	4.49 ± 1.83	4.22[b] (0.35)	10.11	4.60 ± 1.28	4.46[b] (0.64)	7.86	*< 10-4*
Sc (2)	0.166 ± 0.070	0.167[a] (0.050)	0.350	0.133 ± 0.024	0.133[a] (0.000)	0.167	0.195 ± 0.081	0.167[a] (0.05)	0.333	0.294 ± 0.103	0.300[b] (0.067)	0.467	*0.0002*
Sm (2)	0.114 ± 0.027	0.100[a] (0.000)	0.183	0.106 ± 0.012	0.100[a] (0.000)	0.133	0.140 ± 0.051	0.100[a] (0.000)	0.233	0.218 ± 0.083	0.200[b] (0.067)	0.367	*< 10-4*
Th (2)	0.120 ± 0.045	0.100[a] (0.000)	0.233	0.126 ± 0.040	0.100[a] (0.000)	0.200	0.164 ± 0.067	0.167[a] (0.067)	0.300	0.277 ± 0.122	0.233[b] (0.067)	0.633	*< 10-4*
Ti (2)	43.4 ± 17.5	40.0[a] (10.0)	76.7	55.0 ± 12.0	56.7[a] (10.0)	73.3	60.2 ± 21.2	58.3[a] (13.3)	103.3	106.6 ± 34.2	106.7[b] (26.7)	173.3	*< 10-4*
V (2)	1.20 ± 0.41	1.00[a] (0.00)	2.00	1.41 ± 0.43	1.33[ab] (0.33)	2.00	1.88 ± 0.59	2.00[b] (0.33)	3.00	2.80 ± 0.68	3.00[c] (0.33)	4.33	*< 10-4*
Ba (3)	16.8 ± 8.6	15.7[a] (5.0)	44.0	17.3 ± 7.8	17.3[a] (5.7)	32.0	15.6 ± 3.1	17.2[a] (2.0)	18.7	20.7 ± 9.1	17.0[a] (4.7)	43.0	0.4639
Ca (3)	6248 ± 3889	4567[a] (808)	15700	4902 ± 1331	4600[a] (967)	6967	6481 ± 3069	5550[a] (700)	14967	14306 ± 6902	12067[b] (3300)	33367	*< 10-4*
Co (3)	0.227 ± 0.062	0.200[a] (0.000)	0.400	0.402 ± 0.094	0.400[b] (0.067)	0.567	0.269 ± 0.077	0.250[a] (0.050)	0.400	0.362 ± 0.119	0.333[b] (0.067)	0.600	*< 10-4*
Cr (3)	5.26 ± 0.94	5.00[a] (0.33)	8.17	7.02 ± 1.58	6.00[b] (0.33)	10.00	5.93 ± 0.96	5.67[ab] (0.50)	8.33	6.04 ± 0.77	6.00[b] (0.50)	8.00	*0.0008*
Hf (3)	1.51 ± 0.57	1.43[b] (0.21)	2.74	1.44 ± 0.36	1.40[ab] (0.30)	1.88	1.11 ± 0.39	0.95[a] (0.15)	2.09	1.33 ± 0.39	1.32[ab] (0.18)	2.41	0.0513
K (3)	2934 ± 410	2800[a] (167)	3767	3444 ± 622	3167[b] (367)	4700	3536 ± 397	3567[b] (83)	4667	3696 ± 478	3633[b] (233)	4633	*0.0005*
Mg (3)	688 ± 90	700[a] (83)	833	1056 ± 217	933[c] (167)	1433	731 ± 83	717[a] (67)	900	950 ± 130	933[b] (67)	1267	*< 10-4*
Mn (3)	61.5 ± 27.6	52.7[a] (16.7)	126.5	77.4 ± 25.8	75.0[a] (18.7)	123.3	64.9 ± 21.4	63.0[a] (18.7)	105.0	62.1 ± 50.4	38.3[a] (16.7)	178.0	0.1244
Nd (3)	0.443 ± 0.203	0.400[a] (0.067)	0.850	1.048 ± 1.685	0.533[ab] (0.100)	5.533	0.629 ± 0.252	0.583[ab] (0.083)	1.233	1.122 ± 0.365	1.067[b] (0.300)	1.800	*< 10-4*
Ni (3)	1.17 ± 0.63	0.97[a] (0.11)	2.67	4.46 ± 2.34	4.57[b] (1.47)	9.17	1.83 ± 0.54	1.70[a] (0.27)	2.90	1.77 ± 0.34	1.80[a] (0.17)	2.57	*< 10-4*
P (3)	416 ± 88	427[a] (50)	580	587 ± 255	543[ab] (180)	1077	526 ± 106	495[ab] (53)	717	670 ± 209	620[b] (113)	1233	*0.0005*
Sr (3)	14.3 ± 7.5	12.7[a] (4.2)	36.3	12 ± 2.9	11.3[a] (2.0)	16.3	12 ± 4	10.8[a] (2.0)	21.0	32.2 ± 14.4	27.3[b] (4.3)	75.0	*< 10-4*
Te (3)	0.079 ± 0.039	0.063[a] (0.013)	0.165	0.066 ± 0.019	0.060[a] (0.003)	0.103	0.076 ± 0.03	0.073[a] (0.018)	0.140	0.190 ± 0.101	0.153[b] (0.023)	0.483	*< 10-4*
Y (3)	6.55 ± 4.08	4.90[ab] (0.47)	18.97	5.02 ± 0.74	5.33[a] (0.60)	5.93	5.67 ± 1.54	5.50[a] (0.98)	8.33	7.90 ± 3.47	6.50[b] (1.70)	17.17	*0.0466*
Zr (3)	66.1 ± 26.5	62.6[a] (8.5)	128.9	57.1 ± 9.7	56.7[a] (7.4)	68.3	51.2 ± 16.1	49.0[a] (13.6)	76.7	55.4 ± 18.4	52.5[a] (7.8)	109.4	0.2392

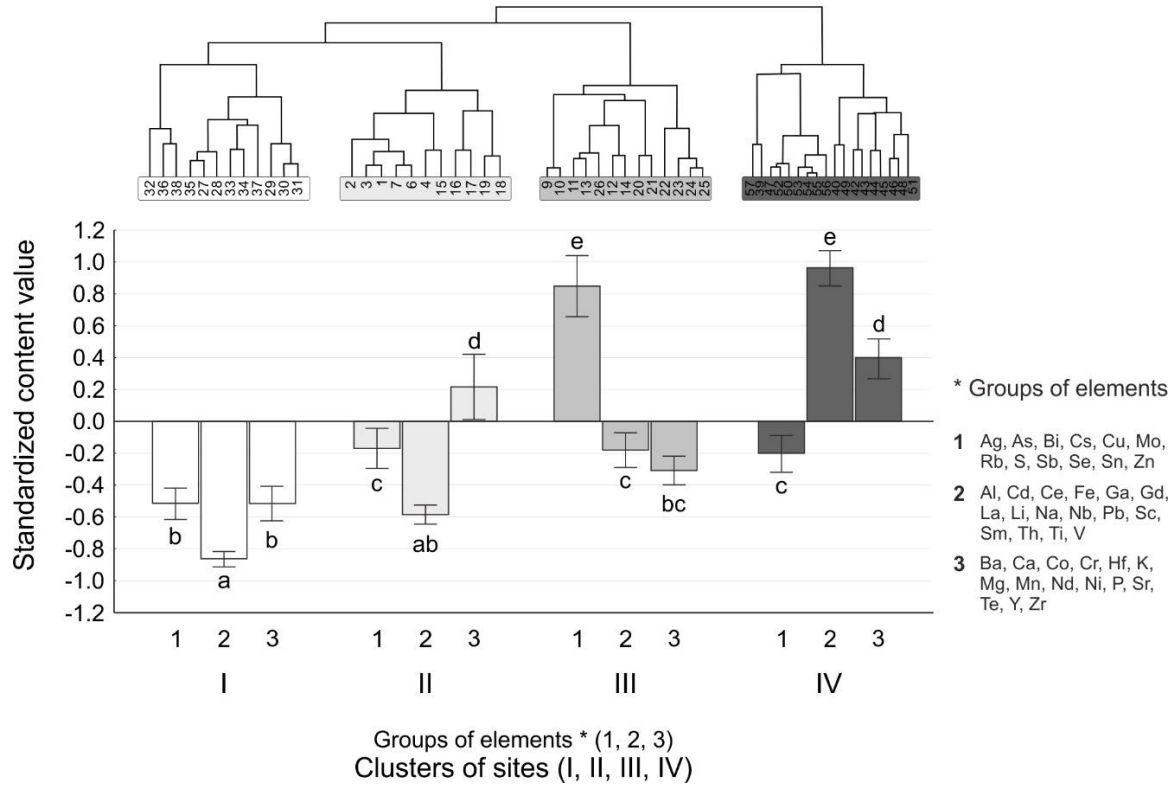


Fig. 3 Content of element groups in the site clusters I-IV: data are separately standardized for each element and showed as mean and 95% confidence interval for 3 different element groups, as resulting from CA. Letters above bars indicate significant pair-wise differences (Tukey's HSD test for unequal sample size, $p < 0.05$).

3.5 Background element content of *Pseudevernia furfuracea*: final remarks

Previous results on *Pseudevernia furfuracea* BEC, as obtained by Cecconi et al. (2018) by *aqua regia* digestion, indicated a pattern of association between *P. furfuracea* BECs and the geographical location of Italian remote sites. The results based on total digestion here presented, clearly confirm the same general pattern. Consistently, elemental content generally increases moving southwards, with increasing averaged levels of population density and agricultural landcover in the surroundings of the sampling sites, and contextual decreasing mean annual rainfall and forest landcover. Among the Alpine sites, elemental content decreases moving eastwards (with increasing precipitation levels), whereas western sites (with prevalent siliceous and/or metamorphic bedrocks) showed higher background levels with respect to the easternmost ones (with prevalent sedimentary bedrocks) (Cecconi et al. 2018). The single cluster obtained for Apennine sites is likely the result of site under-sampling in such a large geographical domain, however the utmost differences still observed between Alpine and Apennine *P. furfuracea* BECs are certainly not a sampling artifact. Indeed, Apennine sites showed higher content of lithogenic (Al, Ca, Fe, Li, Th and Ti) and rare earth elements (REE: Ce, La, Sc, Y), as related to higher soil susceptibility to erosion and influence by agricultural areas (Capozzi et al. 2016; Cecconi et al. 2018).

Compared to BECs based on partially-digested samples, some interesting differences arose. In particular, the Alpine sites segregated into three (instead of two) clusters, substantially differing in elemental content (Table 2, Fig. 3): (*i*) the eastern sites of cluster I, showing the lowest BEC values; (*ii*) the western Alpine sites, with intermediate BEC values, and (*iii*) the central sites of cluster III, located in Valtellina (province of Sondrio) and Adige Valley (provinces of Trento and Bolzano),

with the highest content of some elements of anthropogenic origin (e.g., Zn; Li et al. 2017), long-range transported (i.e., As, Pb; Bargagli 1998), or occurring in several mineralogical assemblages of metamorphic substrates (i.e., Cu, Mo, Rb and Sb; Salminen et al. 2005; Kuleshov 2016). Interestingly, the metamorphic substrates of site clusters II and III were substantially different, with sites of cluster II mostly laying over metamorphosed peridotite and gneiss, and in proximity of serpentinized ultramafic rocks (likely explaining the high contents of Ni and Mg; Aziz et al. 2015), and those of cluster III over phyllite and micaschists. Besides highlighting finer lithological differences, such pattern also reflects differences in anthropization in the Alpine ranges. Indeed, the BEC revealed in the three clusters well matches with differences in population density in the surroundings of the study sites, when calculated from national census data within circular buffers of 25 km radius centred in the sampling sites, as described in Cecconi et al. (2018). As a matter of fact, population density showed utmost between-cluster differences, with a minimum in cluster I (24.2 ± 10.0 inhabitants km^{-2}), a maximum in cluster III (95.9 ± 21.2 inhabitants km^{-2}), and intermediate values in cluster II (67.2 ± 16.4 inhabitants km^{-2}), also reflecting the impact of relevant winter tourism in the surveyed areas.

The limited discrepancies between the two sets of BEC values can be explained by the interplay of several factors, including the acid digestion, different sets of biological replicates and chemical elements analysed. However, despite the above-mentioned local differences, the overall large-scale pattern emerged from the multivariate analysis of totally-digested samples definitively confirmed the robustness of the BEC assessment of Cecconi et al. (2018), based on extensive, purposely devoted field sampling.

4. Conclusions

The selection of a specific acid digestion to be applied in a biomonitoring survey can be a thorny issue which should not be underrated, as possibly leading to under-/overestimation of the content of several elements. As such, this analytical step should always be taken into account as a possible source of bias in the interpretation of bioaccumulation results, especially for elements of particular environmental concern such as As, Ba, Cd, Cu, Ni and Zn. Depending on the specific aim of the biomonitoring survey and the target set of chemical elements, different mineralizations may produce satisfactory recoveries. The acid digestion with hydrofluoric acid was proved to produce more accurate results for the majority of tested elements, especially for Al, Ba, Cu, Fe, Mn, and Zn. In this light, when addressing these elements in biomonitoring applications, a partial digestion should be disregarded in favour of a digestion with hydrofluoric acid. Moreover, when a specific digestion method is adopted in routine biomonitoring studies, this should be clearly stated, and accuracy results should be fully provided, in order to allow easier and more robust cross-study comparisons.

Finally, the background levels were explored for 43 elements after total mineralization of samples of *Pseudevernia furfuracea*. BEC patterns assessed at very large scale, and based on a large amount of field samples, were proved to be rather conservative. The context-dependency of national BEC values was confirmed by identifying geographically separated site clusters for which further BEC values are now available. In this way a major methodological gap in biomonitoring procedures

was filled by providing a complete set of BEC values based on different acid digestions and one of the most performing analytical techniques for multi-element determination, to be alternatively used as a reference according to the selected mineralization procedure.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLES S1-S2

Supplementary Table S1. List of the sampling sites of the epiphytic lichen *Pseudevernia furfuracea*, with location (region; municipality; UTM coordinates, datum WGS84), altitude, collectors, and number of analytical replicates subjected to partial (P) and total (T) digestion methods.

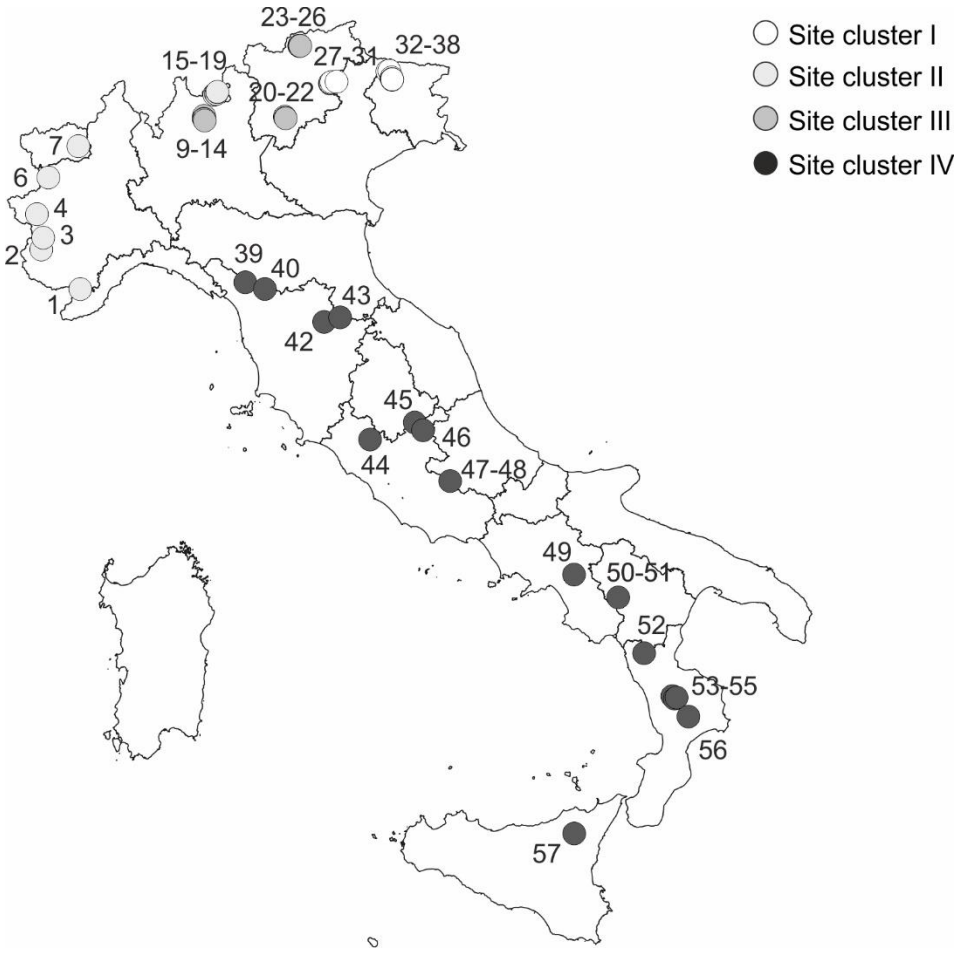
Site ID	Region (province)	Municipality	UTM (E)	UTM (N)	Altitude (m a.s.l.)	Collector	Replicates (P)	Replicates (T)
1	Piemonte (CN)	Briga Alta	396627	4884898	1800	Favero-Longo S.E.		3
2	Piemonte (CN)	Sampeyre	355214	4936081	1400	Favero-Longo S.E.	3	3
3	Piemonte (CN)	Ostana	357617	4959764	1600	Favero-Longo S.E.		3
4	Piemonte (TO)	Perrero	353312	4975156	1560	Bidussi M., Capozzi F.	3	3
5	Piemonte (TO)	Perrero	352934	4975870	1300	Bidussi M., Capozzi F.		3
6	Piemonte (TO)	Groscavallo	364072	5025600	1175	Favero-Longo S.E.	3	3
7	Val d'Aosta (AO)	Challand Saint Anselme	401280	5064495	1550	Favero-Longo S.E.	3	3
8	Val d'Aosta (AO)	Morgex	344106	5066088	1550	Favero-Longo S.E.		3
9	Lombardia (SO)	Fusine	558229	5106887	1205	Capozzi F., Panepinto F.	3	3
10	Lombardia (SO)	Fusine	558929	5103665	1610	Capozzi F., Panepinto F.		3
11	Lombardia (SO)	Fusine	558949	5104594	1425	Capozzi F., Panepinto F.		3
12	Lombardia (SO)	Fusine	558689	5101601	2000	Capozzi F., Panepinto F.	3	3
13	Lombardia (SO)	Fusine	559195	5101932	1820	Capozzi F., Panepinto F.		3
14	Lombardia (BG)	Foppolo	559576	5099879	1950	Capozzi F., Panepinto F.	3	3
15	Lombardia (SO)	Lanzada	569126	5125647	1250	Capozzi F., Panepinto F.		3
16	Lombardia (SO)	Lanzada	570601	5126912	1530	Capozzi F., Panepinto F.		3
17	Lombardia (SO)	Lanzada	571172	5126457	1750	Capozzi F., Panepinto F.		3
18	Lombardia (SO)	Lanzada	572129	5128037	2120	Capozzi F., Panepinto F.		3
19	Lombardia (SO)	Lanzada	572158	5128410	1990	Capozzi F., Panepinto F.	3	3
20	Trentino Alto Adige (TN)	Trento	657706	5100843	1170	Cristofolini F.		3
21	Trentino Alto Adige (TN)	Trento	658302	5099679	1635	Cristofolini F.		3
22	Trentino Alto Adige (TN)	Trento	658562	5099302	1780	Cristofolini F.	3	3
23	Trentino Alto Adige (BZ)	San Leonardo in Passiria	675604	5189316	1720	Candotto Carniel F., Craighero T.		3
24	Trentino Alto Adige (BZ)	San Leonardo in Passiria	675848	5188959	1635	Candotto Carniel F., Craighero T.		3
25	Trentino Alto Adige (BZ)	San Leonardo in Passiria	676278	5189427	1845	Candotto Carniel F., Craighero T.	3	3
26	Trentino Alto Adige (BZ)	San Leonardo in Passiria	676711	5189071	1820	Candotto Carniel F., Craighero T.		3
27	Veneto (BL)	Rocca Pietore	721351	5144028	1965	Bidussi M., Capozzi F.	3	3
28	Veneto (BL)	Rocca Pietore	721655	5144322	1835	Bidussi M., Capozzi F.		3
29	Veneto (BL)	Rocca Pietore	721851	5144747	1660	Bidussi M., Capozzi F.	3	3
30	Veneto (BL)	Rocca Pietore	722894	5145358	1470	Bidussi M., Capozzi F.	3	3
31	Veneto (BL)	Rocca Pietore	727887	5145740	1180	Bidussi M., Capozzi F.		3
32	Veneto (BL)	Vigo di Cadore	778437	5155969	1540	Capozzi F., Panepinto F.		3
33	Friuli Venezia Giulia (UD)	Prato Carnico	780331	5156611	1295	Capozzi F., Panepinto F.		3
34	Friuli Venezia Giulia (UD)	Ampezzo	782515	5149030	1460	Capozzi F., Panepinto F.		3
35	Friuli Venezia Giulia (UD)	Forni di Sotto	782592	5149904	1275	Capozzi F., Panepinto F.		3
36	Friuli Venezia Giulia (UD)	Ampezzo	782896	5148406	1650	Capozzi F., Panepinto F.	3	3
37	Friuli Venezia Giulia (UD)	Ampezzo	783063	5147585	1970	Capozzi F., Panepinto F.		3
38	Friuli Venezia Giulia (UD)	Ampezzo	783186	5148045	1850	Capozzi F., Panepinto F.		3
39	Toscana (LU)	San Romano in Garfagnana	608580	4895302	1230	Benesperi R.		3
40	Toscana (PT)	Abetone	632906	4887038	1315	Benesperi R.		3
41	Toscana (PT)	Abetone	633384	4889793	1425	Benesperi R.		3
42	Toscana (FI)	Reggello	706379	4846165	1130	Benesperi R.	3	3
43	Toscana (AR)	Poppi	726310	4851715	960	Benesperi R.	3	3
44	Lazio (VT)	Soriano nel Cimino	763440	4700006	1060	Ravera S.		3
45	Umbria (TR)	Polino	818942	4721156	1400	Ravera S.	3	3
46	Lazio (RI)	Leonessa	829212	4711427	1620	Ravera S.		3
47	Lazio (FR)	Filettino	862329	4649914	1770	Ravera S.		3
48	Lazio (FR)	Filettino	862883	4648429	1600	Ravera S.	3	3
49	Campania (AV)	Bagnoli Irpino	1016725	4532218	1490	Capozzi F.	3	3
50	Basilicata (PZ)	Sasso di Castalda	1071257	4504090	1615	Potenza G., Romano A.	3	3
51	Basilicata (PZ)	Abriola	1071592	4503849	1615	Potenza G., Romano A.		3
52	Calabria (CS)	Morano Calabro	1103640	4434506	1335	Puntillo D.		3
53	Calabria (CS)	Celico	1138732	4381258	1430	Puntillo D.		3
54	Calabria (CS)	Spezzano Piccolo	1141751	4378020	1650	Puntillo D.		3
55	Calabria (CS)	Spezzano della Sila	1144011	4379400	1440	Puntillo D.		3
56	Calabria (CZ)	Taverna	1158747	4355740	1600	Puntillo D.		3
57	Sicilia (CT)	Randazzo	1016912	4210511	960	Carasci A., Cataldo D.		3

Supplementary Table S2. Mean recovery percentages and 95% confidence interval (95% C.I.) of element content in the in-house standard reference materials (plant leaves CDV-1 and V16, for partial digestion; soil OREAS25A-4A and OREAS45E for total digestion). Mean recoveries and confidence interval were calculated on 11 and 5 analytical replicates for in-house standards of packets VG101-EXT and MA250, respectively.

Element	**Total digestion** (MA250; n = 5)		**Partial digestion** (VG101-EXT; n=11)	
	OREAS25A-4A	**OREAS45E**	**CDV-1**	**V16**
Ag	-	108.7 (101.2 ÷ 116.3)	109.1 (97.6 ÷ 120.6)	115.6 (109.2 ÷ 122.1)
Al	104.7 (99.8 ÷ 109.5)	105.1 (102.3 ÷ 107.9)	100.6 (95.1 ÷ 106.1)	104.1 (97.2 ÷ 111)
As	-	111.9 (103.6 ÷ 120.2)	97.2 (85.1 ÷ 109.3)	93.8 (85.1 ÷ 102.4)
Au*	-	-	91.7 (55.5 ÷ 127.9)	125.3* (96.7 ÷ 153.8)
B	-	-	99.2 (94.6 ÷ 103.9)	101.8 (97.8 ÷ 105.9)
Ba	111.3 (104.6 ÷ 118)	106.5 (102.2 ÷ 110.8)	102.1 (99 ÷ 105.2)	106.7 (101.9 ÷ 111.5)
Be	98 (98 ÷ 98)	-	-	-
Bi	105.1 (94.4 ÷ 115.9)	102.9 (93.2 ÷ 112.5)	118.2 (77.7 ÷ 158.7)	-
Ca	94.5 (86.8 ÷ 102.2)	104.6 (96.1 ÷ 113.2)	104.5 (101.7 ÷ 107.4)	108.5 (105.6 ÷ 111.4)
Cd*	-	76.7* (58.2 ÷ 95.2)	93.2 (85.3 ÷ 101)	98.3 (90.2 ÷ 106.4)
Ce	107.2 (104.2 ÷ 110.2)	111 (106.9 ÷ 115.1)	110.2 (106.4 ÷ 114.1)	103.6 (97.4 ÷ 109.8)
Co	98.5 (94.6 ÷ 102.5)	105.4 (100.3 ÷ 110.5)	99.2 (95 ÷ 103.5)	95.5 (86.2 ÷ 104.8)
Cr	104.3 (97.9 ÷ 110.8)	107.1 (101.9 ÷ 112.3)	111.6 (104.6 ÷ 118.6)	95.7 (84 ÷ 107.3)
Cs	98.5 (93.3 ÷ 103.6)	104.8 (96.5 ÷ 113)	103.2 (97.3 ÷ 109.1)	103.8 (99.9 ÷ 107.6)
Cu	103.8 (96.3 ÷ 111.2)	103.5 (99.1 ÷ 107.8)	101.2 (97.3 ÷ 105)	103 (96 ÷ 109.9)
Dy	-	105.4 (91.4 ÷ 119.3)	-	-
Er	-	95 (89.3 ÷ 100.7)	-	-
Eu	-	119.2 (99.3 ÷ 139.2)	-	-
Fe	101 (96.8 ÷ 105.1)	104 (101.4 ÷ 106.7)	114.6 (109.4 ÷ 119.8)	107.9 (95.5 ÷ 120.3)
Ga*	105.6 (101.5 ÷ 109.8)	105.1 (100 ÷ 110.1)	110.9 (103.9 ÷ 117.9)	68.2* (51.2 ÷ 85.1)
Gd	-	115.4 (93.3 ÷ 137.5)	-	-
Ge*	-	-	60.6* (36.4 ÷ 84.8)	110.9 (87.4 ÷ 134.5)
Hf	98.4 (92.7 ÷ 104.2)	100.4 (90.5 ÷ 110.3)	100 (89.4 ÷ 110.6)	101.5 (72.6 ÷ 130.5)
Hg	-	-	107.3 (98.9 ÷ 115.7)	116.9 (108.6 ÷ 125.1)
Ho	-	94.7 (76.8 ÷ 112.6)	-	-
In	-	107.1 (86.1 ÷ 128.1)	-	-
K	106.6 (96.1 ÷ 117.2)	111.7 (102.6 ÷ 120.9)	106.1 (102.5 ÷ 109.6)	110.7 (109.2 ÷ 112.3)
La*	105.9 (101.1 ÷ 110.6)	107.1 (100.2 ÷ 114)	109.5 (107 ÷ 112)	76.4* (66.3 ÷ 86.5)
Li	107.1 (100.5 ÷ 113.7)	109.4 (105.6 ÷ 113.2)	107 (101.4 ÷ 112.5)	94.8 (82.5 ÷ 107.2)
Lu	-	102.9 (71.1 ÷ 134.6)	-	-
Mg	104.6 (100.4 ÷ 108.7)	102.6 (96.9 ÷ 108.2)	104.7 (102.6 ÷ 106.8)	106.3 (103.8 ÷ 108.9)
Mn	107.4 (101.9 ÷ 112.8)	98.6 (95 ÷ 102.2)	104.3 (102.2 ÷ 106.4)	100 (98.2 ÷ 101.7)
Mo	98.2 (90.3 ÷ 106.1)	97.6 (91.4 ÷ 103.8)	103.6 (97.8 ÷ 109.5)	108.6 (94.1 ÷ 123.2)
Na	101.5 (93 ÷ 110)	98.6 (92.1 ÷ 105.2)	110.1 (104.1 ÷ 116.2)	90.9 (68.3 ÷ 113.5)
Nb	93.5 (87.6 ÷ 99.4)	-	105.5 (96.8 ÷ 114.1)	90.9 (76.7 ÷ 105.1)
Nd	-	110.7 (100.7 ÷ 120.7)	-	-
Ni	107.2 (101.1 ÷ 113.4)	107.4 (103.5 ÷ 111.3)	105.5 (101.2 ÷ 109.9)	106.5 (93 ÷ 120)
P	109.2 (100.9 ÷ 117.5)	103.5 (96.5 ÷ 110.6)	104.5 (100.5 ÷ 108.6)	98.5 (96.5 ÷ 100.6)
Pb	102 (91.5 ÷ 112.6)	107.9 (99.4 ÷ 116.5)	99.5 (95.3 ÷ 103.6)	97.1 (94.7 ÷ 99.5)
Pr	-	110.1 (98.1 ÷ 122.1)	-	-
Rb	102.6 (97.9 ÷ 107.3)	109.6 (104.2 ÷ 115.1)	111.5 (107.7 ÷ 115.2)	99.5 (96.7 ÷ 102.2)
Re	-	-	-	-
S*	98 (98 ÷ 98)	87.0 (87.0 ÷ 87.0)	150.0* (131.7 ÷ 168.3)	303.0* (267.9 ÷ 338.1)
Sb	95.2 (84.2 ÷ 106.2)	106.4 (95.3 ÷ 117.5)	84.8 (69.5 ÷ 100.2)	84.4 (77.7 ÷ 91.1)
Sc	93.9 (89 ÷ 98.8)	100.8 (95.4 ÷ 106.2)	113.6 (104.9 ÷ 122.4)	-
Se*	-	94.3 (70.4 ÷ 118.1)	72.7* (55.9 ÷ 89.5)	-
Sm	-	99.1 (84.9 ÷ 113.3)	-	-
Sn	104.9 (95.6 ÷ 114.3)	118.2 (103.9 ÷ 132.4)	125 (118.5 ÷ 131.5)	100.8 (88.3 ÷ 113.3)
Sr	96.9 (85.0 ÷ 108.8)	106.9 (99.1 ÷ 114.7)	102.9 (100.9 ÷ 104.9)	100.3 (98.3 ÷ 102.4)
Ta	93.8 (88.3 ÷ 99.2)	103.7 (91.1 ÷ 116.3)	-	-
Tb*	-	72.7* (52.1 ÷ 93.3)	-	-
Te*	-	130* (76.6 ÷ 183.4)	-	-
Th	101.4 (92.8 ÷ 110)	106 (100.5 ÷ 111.6)	105.8 (99.9 ÷ 111.8)	-
Ti	100.6 (94.7 ÷ 106.5)	100.3 (92.9 ÷ 107.7)	99.7 (93.9 ÷ 105.5)	90.9 (87.9 ÷ 93.9)
Tl*	108 (97.2 ÷ 118.8)	191.1* (168.4 ÷ 213.8)	-	-
Tm	-	94.1 (54.1 ÷ 134.1)	-	-
U	96.6 (92.8 ÷ 100.4)	109.5 (100.9 ÷ 118.2)	97.9 (93.1 ÷ 102.6)	-
V*	102.4 (98.9 ÷ 105.9)	103.1 (99.2 ÷ 107)	220.8* (208.2 ÷ 233.4)	-
W	97.1 (88.2 ÷ 106.1)	100.9 (91.2 ÷ 110.6)	-	-
Y	84.7 (78.1 ÷ 91.4)	-	109.7 (107 ÷ 112.4)	116.1 (108.1 ÷ 124)
Yb	-	107.4 (100.2 ÷ 114.7)	-	-
Zn	102 (92.7 ÷ 111.3)	102.6 (98.2 ÷ 107.1)	96.9 (94.1 ÷ 99.7)	99.5 (97.8 ÷ 101.3)
Zr	-	106.6 (96.8 ÷ 116.4)	107.1 (101.7 ÷ 112.6)	99 (88.9 ÷ 109.1)

* Mean recoveries either below 80% or above 120%.

SUPPLEMENTARY FIGURE S1



Supplementary Figure S1. Map of field sites (ID codes and details in Supplementary Table S1) symbolized according to CA results (Fig. 2A).

New interpretative scales for lichen bioaccumulation data: The Italian proposal

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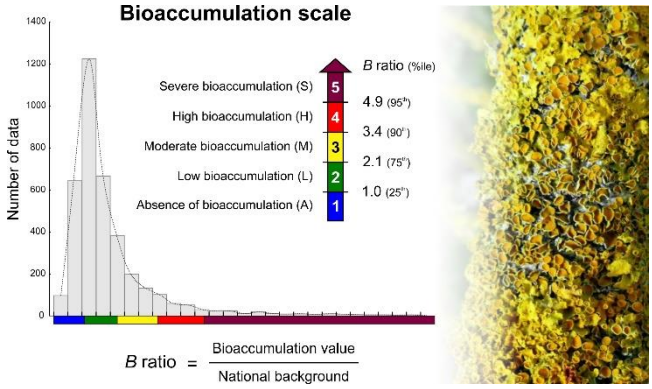
Abstract

The interpretation of lichen bioaccumulation data is of paramount importance in environmental forensics and decision-making processes. By implementing basic ideas underlying previous interpretative scales, new dimensionless, species-independent "bioaccumulation scales" for native and transplanted lichens are proposed. Methodologically consistent element concentration datasets were populated with data from biomonitoring studies relying on native and transplanted lichens. The scale for native lichens was built up by analyzing the distribution of ratios between element concentration data and species-specific background concentration references (*B* ratios), herein provided for *Flavoparmelia caperata* and *Xanthoria parietina* (foliose lichens). The scale for transplants was built up by analyzing the distribution of ratios between element concentration in exposed and unexposed samples (*EU* ratio) of *Evernia prunastri* and *Pseudevernia furfuracea* (fruticose lichens). Both scales consist of five percentile-based classes; namely, "Absence of", "Low", "Moderate", "High", and "Severe" bioaccumulation. A comparative analysis of extant interpretative tools showed that previous ones for native lichens suffered from the obsolescence of source data, whereas the previous expert-assessed scale for transplants failed in describing noticeable element concentration variations. The new scales, based on the concept that pollution can be quantified by dimensionless ratios between experimental and benchmark values, overcome most critical points affecting the previous scales.



Keywords: biomonitoring; native lichens; lichen transplants; air pollution; trace elements; background levels; *Flavoparmelia caperata*; *Xanthoria parietina*; *Evernia prunastri*; *Pseudevernia furfuracea*.

1. Introduction

Air quality standards are fundamental references in environmental policy. These standards are mostly set up from data on atmospheric pollutant concentrations obtained by continuous measurements within fully- or semi-automatic apparatus (Bargagli et al. 1997). Nevertheless, the need for evaluating biological effects of low concentrated air pollutants over large geographical areas has made biomonitoring of paramount importance to provide and integrate information on pollutant depositions (Bargagli 1998; Ferretti and Erhardt 2002). A fortiori, this is true for trace element pollution. In this respect, the extensive use of lichens as effective bioaccumulators has provided valuable data through the years (Brunialti and Frati 2014). A crucial point with potential outcomes for decision making and environmental forensics is the appropriate interpretation of biomonitoring results (Brunialti et al. 2004). This issue has been extensively addressed in the context of human biomonitoring and chemical risk assessment (e.g., Hays et al. 2007; Clewell et al. 2008), but it has also been faced in the field of biomonitoring by mosses and lichens. Indeed, several efforts have been made to achieve high quality standards for the moss bag technique, hence to improve cross-study comparison and guarantee proper result interpretation (Giordano et al. 2013; Capozzi et al. 2016). Even for lichens, efforts have been made to enhance methodological standardization and result interpretation. In particular, in this case, interpretative tools were purposely developed for bioaccumulation data from both native and transplanted lichens.

In biomonitoring techniques based on native lichens (see Table 1 for a glossary), measured element concentrations are usually compared with background element concentration values (BECs; Table 1) and, when these are not available, results are expressed as deviations from the minimum values revealed in the study area, considered as an "internal baseline" (Bargagli and Nimis 2002). BECs can either be obtained by analyzing literature data (review-based BECs, (Bargagli 1998; Bennett 1999) or assessed by direct large-scale field campaigns (field-assessed BECs; Bargagli et al. 1999; Monaci et al. 2012; Cecconi et al. 2018). Both approaches should follow robust methodological guidelines and, in fact, the discussion on their proper assessment in lichen matrices is of current interest (Cecconi et al. 2018; Cecconi et al. 2019). Another approach to the interpretation of biomonitoring data from native lichens is the use of interpretative scales (Nimis and Bargagli 1999) based on thresholds identifying classes of increasing element concentrations, and obtained by the meta-analysis of a large set of bioaccumulation data for epiphytic lichens at the national level (Nimis et al. 2001). The so-called "naturality/alteration scales", extensively applied until now (e.g., Brunialti and Frati, 2007; Demiray et al. 2012; Pirintsos et al. 2014; Cocozza et al. 2016; Paoli et al. 2017; Lucadamo et al. 2018), were originally proposed by Nimis and Bargagli and consist of seven classes of element concentrations built up on hundreds of data points collected in Italy between the 1980s and the 1990s (Nimis and Bargagli 1999). Source data referred to 17 elements with a minimum of 100 records each, obtained from at least three biomonitoring surveys carried out in areas characterized by different pollution levels and geomorphology (Nimis and Bargagli 1999). The simple core idea behind these interpretative scales is undoubtedly powerful. However, as recognized by the same authors, some important issues remained unsolved. In particular, naturality/alteration scales are multi-specific, meaning that the source data referred to manifold lichen species, hence posing an important problem related to the acknowledged species-

specificity of lichen bioaccumulation (Nimis et al. 2001; Tretiach et al. 2001; Minganti et al. 2003; Bergamaschi et al. 2007). Moreover, in the intentions of authors, source data should have been reported in an accessible database, so as to allow the inclusion of new records together with important methodological information (e.g., lichen species, geographical location, sample mineralization technique). Unfortunately, this did not occur and the same source data on which the scales were built up remained unpublished.

For biomonitoring techniques based on lichen transplants (Table 1), the interpretation is generally based on the comparison of the elemental concentrations measured in samples exposed in the target study area for 0.5–6(–12) months and those measured in unexposed samples (Table 1) immediately after collection of the bulk material in a proximate-natural site. Even in this case, an interpretative scale is available (Frati et al. 2005). The core idea behind this scale, originally proposed by Frati et al. (Frati et al. 2005), was substantially different from that of naturality/alteration scales, because the data are expressed as a ratio, the so-called exposed-to-control (*EC*) ratio (Table 1), calculated by dividing the element concentration values of exposed samples (eventually the mean values, if more samples are exposed at the same site) by those of unexposed samples. The resulting "accumulation/loss scale" consisted of five classes built up on arbitrary cutoffs (that is, progressive ±25% deviations from the unitary *EC* value; Frati et al. 2005) on the basis of considerations derived from lichen bioindication studies (Loppi et al. 2002; Frati et al. 2005).

Table 1. Glossary of main terms and concepts.

Glossary	
Native lichens	Lichens grown in a target study area.
Background element concentration values (BECs)	Species-specific element concentration values measured in lichen samples reflecting proximate-natural, unaltered conditions.
Bioaccumulation ratio (*B* ratio)	The dimensionless ratio between species-specific element concentration values measured in native samples and the corresponding background values.
Lichen transplants	Lichens collected in a proximate-natural site and afterwards transplanted for a certain exposure time span to a target study area.
Exposed samples	In the context of lichen transplants, samples transplanted to a target study area, exposed to pollutant depositions for a certain exposure time span, and then subjected to the determination of elemental concentration.
Unexposed samples	In the context of lichen transplants, samples collected in a proximate-natural site and subjected to the determination of elemental concentration. These samples are used as a benchmark to assess the magnitude of lichen bioaccumulation after transplantation.
Exposed-to-unexposed ratio (*EU* ratio)	The dimensionless ratio between species-specific element concentration values measured in exposed samples and the corresponding element concentration values measured in unexposed samples.
Exposed-to-control ratio (*EC* ratio)	Previous name of the *EU* ratio (27), here terminologically revised.

The aim of this work was to develop new scales, using a methodologically consistent pipeline and revised terminology, based on the meta-analysis of biomonitoring data. In particular, we conceptualized two dimensionless scales for native and transplanted lichens, based on (*i*) the ratio between element concentration data and species-specific review-based BECs (herein contextually provided), and (*ii*) the ratio between element concentration values measured in exposed and unexposed lichen samples, respectively. Both scales, along with the previously available ones, were also applied to real case studies in order to assess their relative performance. The new scales are

valid for Italy, but being based on a robust conceptual framework, they may easily be implemented in other countries.

2. Data and Methodology

2.1. Data collection

A literature search was undertaken between April and May 2018, in order to compile a list of eligible biomonitoring studies targeting lichens as bioaccumulators of trace elements, with the main aim of populating two datasets, including (*i*) bioaccumulation data from biomonitoring studies relying on native lichens (herein, dataset *N*: i.e., Native) and (*ii*) bioaccumulation data from biomonitoring studies relying on lichen transplants (herein, dataset *T*: i.e., Transplants). References were included when based on (a) native lichens, referring to thalli grown at different environmental conditions, from proximate-natural to variously human-impacted ones; and (b) lichen transplants, referring to lichen material purposely collected in areas unaffected by significant levels of airborne pollutants and afterwards exposed in polluted areas for relatively short periods. Studies were then reviewed and excluded when meeting at least one of the following conditions: (*i*) the study was carried out outside the Italian territory; (*ii*) data were pooled for different lichen species; or (*iii*) transplanted samples were exposed for more than four months (non-routine exposure time spans).

Element concentration values were recorded just as reported in the papers, with the exception of values below the limit of instrumental detection (LOD), which were recorded as LOD values. All values were expressed in μg g^{-1} dry weight (DW). In addition to the element concentration data, methodological information concerning the acid sample digestion was also recorded, because such a procedure is known to affect the results of elemental analytical determination (Baffi et al. 2002; Yafa and Farmer 2006; Cecconi et al. 2019). Moreover, relevant information concerning the lichen species, the administrative region of study areas, and the year of data collection or publication (when the former was missing) was recorded as well. Data from lichen transplants were labelled according to their type: (*i*) element concentration data from unexposed samples, and (*ii*) element concentration data from exposed samples. The exposure time span of transplants was also recorded, using the week as base unit.

2.2. Data processing

The dataset *N* was initially subjected to a methodological screening. In order to enhance data homogeneity, element concentration data obtained with partial acid digestion of lichen samples (i.e., without hydrofluoric acid, HF) were discarded to enhance methodological uniformity and because this mineralization approach, although largely used and safer for operator health, may determine unsatisfactory recoveries for typical tracers of soil contamination (Tam and Yao 1999; Cecconi et al. 2019). Moreover, data before 2008 were also removed (temporal data filtering) to increase methodological comparability. Indeed, older biomonitoring studies were often deficient in methodological details concerning sample processing procedures, with special reference to sample cleaning (i.e., washing vs. manual cleaning) (Cecconi et al. 2018) and selection of suitable parts of thalli (i.e., peripheral parts vs. whole thalli) (Fortuna et al. 2018), all procedures known to bias the lichen elemental concentration (Nimis et al. 2001). Also, when these studies reported such

information, a substantial methodological heterogeneity was highlighted. By contrast, in the most recent literature, sample washing and the use of whole thalli were abandoned in favor of a manual debris cleaning and the use of peripheral portions of thalli. In addition, all the elements with data deriving from lichen samples collected from less than three administrative Italian regions were excluded, along with those characterized by less than 40 records. Afterwards, the dataset was carefully screened for the occurrence of duplicated records, typing errors, or inconsistent units of measurements. Descriptive statistics were finally calculated for different elements and lichen species; these included the number of records, mean, median, range, quartiles, as well as skewness and kurtosis of the element concentration data distribution.

The construction of the dataset *N* enabled easy assessment of review-based, methodologically uniform background element concentration values (BECs) for frequently used species. Indeed, following the rank-based approach used for the assessment of quality levels of soils and sediments (Gaudet et al. 2001), the 25th percentile of species- and element-specific bioaccumulation data distributions was selected as a background benchmark, and each value below this threshold was regarded as a result of unpolluted conditions. Descriptive statistics, that is, mean, standard deviation, median, and median absolute deviation (Reimann and Garrett 2005), were provided for the sub-dataset consisting of element concentration values below the BEC threshold (BEC dataset). Median values of the BEC dataset were also tested for inter-specific significant differences using Mann–Whitney's U test for independent samples.

After having identified species-specific BECs as the 25th percentile of dataset *N*, each element concentration value in the same dataset was divided by the corresponding BEC value to obtain a new dataset of the same size that included dimensionless values, namely the ratios between element concentration and background values. This simple procedure was inspired by common practices in soil geochemistry. Indeed, many authors (Facchinelli et al. 2001; Massas et al. 2009; Yang et al. 2009) have suggested that the calculation of ratios of element concentrations observed in topsoil to those in the subsoil may provide a reliable indication of contamination (Reimann and de Caritat 2017). Moreover, this approach is methodologically similar to that used for the expression of results in transplant-based studies (see infra). Such a unitless entity, the *B* ratio (i.e., Bioaccumulation ratio; Table 1), indicates absence of bioaccumulation with respect to the national background when it is lower than or equal to 1, whereas it indicates bioaccumulation occurrence when it exceeds 1. *B* ratios, organized in a single column vector (i.e., *B* ratio dataset; Supplementary Data S1), were then tested for significant inter-specific differences (Supplementary Methods S1; Supplementary Table S1). Finally, skewness and kurtosis of the *B* ratio distribution were calculated. After appraisal of *B* ratio distributional shape, the 25th, 75th, 90th, and 95th percentiles were used as interval thresholds to define a five-class interpretative scale.

The dataset *T* was also subjected to a preliminary methodological screening; data obtained with partial acid digestion of lichen samples were discarded. However, because the dataset was far smaller than the dataset *N*, no temporal data filtering was performed, and only those elements characterized by less than 25 records were removed.

Each element concentration value referring to exposed samples in the dataset *T* was divided by the corresponding unexposed mean value, so as to obtain a new dataset that included dimensionless values, namely the ratios between element concentration values of exposed and unexposed lichen

samples. This further unitless entity, the *EU* ratio (i.e., exposed-to-unexposed ratio; Table 1), previously proposed by Frati et al. (2005) as *EC* ratio, and herein terminologically revised, indicates absence of bioaccumulation with respect to a local unaltered situation when it is lower than or equal to 1, whereas it indicates bioaccumulation occurrence when it exceeds 1. *EU* ratios, organized in a single column vector, were then tested for significant inter-specific differences, as done for the *B* ratios of native lichens (Supplementary Methods S1; Supplementary Table S1). *EU* ratio data were analyzed to assess the most frequent exposure time spans (expressed in weeks). Data obtained from the analysis of samples exposed for commensurable time spans were then uniformly labelled (e.g., 8 and 9 week-transplants; Sect. 3.2). Subsequently, data were split into three sub-datasets, homogeneous for transplant exposure time span (i.e., *EU* ratio sub-datasets; Data S2–S4). Each sub-dataset was further screened for upper outliers according to the Tukey method (i.e., values higher than the third quartile of the distribution plus three times the interquartile range), which makes no distributional assumptions and is applicable to skewed or non-bell-shaped data distributions (Hoaglin et al. 1986). After outlier removal, each sub-dataset was further tested for inter-specific differences (Supplementary Methods S1; Supplementary Table S1). Finally, skewness and kurtosis of *EU* ratio distributions were calculated. After appraisal of *EU* ratio distributional shape, the 25^{th}, 75^{th}, 90^{th}, and 95^{th} percentiles were calculated and corrected to account for the overall uncertainty associated with small-sized datasets. In particular, *EU* ratio values were corrected by subtracting from them a percentage corresponding to the semi-range of the 95% confidence interval of *EU* ratio data divided by the mean (Cicchitelli et al. 1992). The corrected-percentiles were then used as interval thresholds to define a five-class interpretative scale, with the exception of the boundary between Class 1 and Class 2, which was aprioristically established at the unitary value because this represents the discernibility threshold between the occurrence of bioaccumulation (*EU* ratio > 1) and its absence (*EU* ratio ≤ 1).

All data analyses and graphics were performed with the software packages Statistica v. 10 (StatSoft Inc., Tulsa, OK, US) and Microsoft Excel (Microsoft Office Professional Plus 2010), with statistical significance tested at $\alpha = 0.05$ in all cases. Figures were edited with CorelDraw X7.

2.3. Working examples: Case studies from NE Italy

The results obtained with the new interpretative scales and with the previous ones for native (Nimis and Bargagli 1999) and transplanted lichens (Frati et al. 2005) were compared using two case studies from NE Italy, respectively obtained by analyzing (a) native samples of *Flavoparmelia caperata* and *Xanthoria parietina* and (b) transplanted samples of *Pseudevernia furfuracea*.

Samples of *F. caperata* and *X. parietina* were collected in 2014 at 40 sampling sites around a coal-fired thermoelectric power plant in the municipality of Monfalcone (NE Italy) (ARPA FVG; Fortuna 2018). Sampling sites were distributed according to a systematic design (regular grid, 2 × 2 km) and, when possible, lichen thalli were collected on the same host tree species. Element concentration (expressed in $\mu g\ g^{-1}$ dry weight (DW)) was measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) after total sample mineralization (ARPA FVG). The severity of pollutant depositions was expressed according to (*i*) naturality/alteration scales (Nimis and Bargagli 1999; Supplementary Table S3) and (*ii*) the bioaccumulation scale for native lichens proposed here. The results reported were limited to As, Cd, and Cr, three elements of environmental and health

concern (EU Directive 2008/507EC; IARC 2018) that were randomly extracted from the set of elements included in the dataset *N*. The random selection was meant to avoid a potential bias due to expert-based selection of elements, possibly leading to a "desired outcome". For this reason, the random extraction prevailed on other criteria, such as the accuracy achieved in the determination of the elemental content. In this respect, As, Cd, and Cr were characterized by recovery percentages of 109%, 70%, and 98%, respectively (ARPA FVG).

For the transplant case study, we referred to ancillary data of a biomonitoring study aimed at evaluating the contamination of mercury around a waste incinerator located in the northern Friulian plane (NE Italy; Tretiach et al. 2011). Samples of *P. furfuracea* were collected in 2008 in a remote area of the eastern Alps and transplanted for 12 weeks to 30 sites distributed along three linear transects centered on the waste incinerator, mostly characterized by agricultural land use (Tretiach et al. 2011). Element concentration (expressed in μg g^{-1} DW) was measured by ICP-MS after total sample mineralization (Tretiach et al. 2011). *EU* ratios were calculated and used to assess the severity of pollutant depositions according to (*i*) the accumulation/loss scale (Frati et al. 2005) and (*ii*) the bioaccumulation scale for lichen transplants proposed here. Even in this case, the results reported were limited to As, Cd, and Cr, for which recovery percentages were 99%, 94%, and 101%, respectively (Tretiach et al. 2011).

Cartographic representations showing sampling and transplant sites and the outcome of the application of different interpretative scales were provided. Cartographic elaborations were performed with QGIS 2.18.27 'Las Palmas'.

3. Results and Discussion

3.1. Native lichens

3.1.1. Source data and species-specific BECs

The dataset *N* included 32,187 bioaccumulation data points from native lichen samples. Element concentration data referred to 42 elements measured in samples of five lichen species collected in 18 administrative Italian regions. Species included *Flavoparmelia caperata*, *Parmelia sulcata*, and *Xanthoria parietina* (foliose lichens), as well as *Evernia prunastri* and *Pseudevernia furfuracea* (fruticose lichens). After the methodological and temporal data filtering, the dataset *N* included 3773 records for 11 elements of environmental concern analyzed in the context of 11 studies (either published or not; in the latter case, methodologically consistent data were provided by the authors; Supplementray Data S1). Data referred to samples of the lichen species *F. caperata* and *X. parietina* (Table 2; Supplementary Fig. S1) collected in five Italian regions (Friuli Venezia Giulia, Lazio, Liguria, Molise, and Toscana).

Table 2. Descriptive statistics of element concentration values included in dataset *N* for *Flavoparmelia caperata* and *Xanthoria parietina*. Statistics refer to the data counts (n), mean and median values (Mean, Med), minima and maxima (Range), interquartile range (IQR), skewness (S), and kurtosis (K). Mean and median values, minima and maxima, as well as interquartile ranges are expressed in μg g^{-1} dry weight (DW) (n.a., data not available).

Element	*Flavoparmelia caperata*							*Xanthoria parietina*						
	n	Mean	Med	Range	IQR	S	K	n	Mean	Med	Range	IQR	S	K
Al	244	551	348	110 - 4224	252 - 526	3.24	11.63	68	656	605	150 - 3408	371 - 722	3.58	16.28
As	367	0.34	0.25	0.06 - 1.90	0.18 - 0.40	2.63	9.08	79	0.35	0.28	0.06 - 2.31	0.15 - 0.40	3.41	14.59

Table 2 (continued)

Element	*Flavoparmelia caperata*							*Xanthoria parietina*						
	n	Mean	Med	Range	IQR	S	K	n	Mean	Med	Range	IQR	S	K
Cd	298	0.30	0.25	0.06 - 1.69	0.18 - 0.37	2.62	13.68	80	0.15	0.09	0.04 - 1.46	0.07 - 0.15	4.73	27.97
Cr	321	2.44	1.84	0.35 - 24.94	1.17 - 2.66	4.80	33.16	77	2.39	1.91	0.69 - 10.52	1.61 - 2.73	2.82	10.87
Cu	321	8.58	7.38	2.50 - 78.29	6.23 - 9.34	6.99	67.44	98	5.83	5.48	3.20 - 19.27	4.45 - 6.31	3.17	13.29
Hg	182	0.09	0.08	0.01 - 0.43	0.06 - 0.11	1.91	8.97	77	0.06	0.05	0.01 - 0.63	0.04 - 0.07	5.55	37.18
Ni	296	3.14	2.67	0.32 - 19.01	1.27 - 4.03	2.61	9.42	51	3.39	2.66	0.82 - 11.2	1.55 - 4.68	1.45	2.68
Pb	321	6.0	4.0	0.8 - 114.2	2.40 - 6.30	7.24	69.56	98	2.37	1.64	0.36 - 15.40	1.00 - 2.67	3.03	11.78
Ti	184	41.8	26.4	0.3 - 309.0	19.4 - 40.7	2.80	9.21	42	59.8	48.9	15.2 - 262.0	37.2 - 60.0	3.00	10.57
V	150	1.71	0.94	0.34 - 13.22	0.75 - 1.60	2.87	10.67	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Zn	321	47.3	44.0	17.7 - 330.8	35.3 - 53.0	6.17	65.08	98	30.0	25.6	13.0 - 168.0	21.2 - 34.4	4.88	33.51

The foliose lichen species *F. caperata* and *X. parietina* are the most used species in biomonitoring based on native lichens across Italy (Brunialti and Frati 2014). Indeed, such species are widespread, with very abundant populations from the submediterranean to the submontane belt (Nimis 2016), providing adequate sampling density for a reliable assessment of pollutant deposition patterns (Bargagli and Nimis 2002). Overall, *F. caperata* and *X. parietina* accounted for 79.6% and 20.6%, respectively, of data. All elements, except for V, included data from both lichen species (Table 2). The methodological data filtering resulted in a substantial reduction of the dataset (−88.3%). However, the final data sizes, separately reported for each element and species, were comparable to those reported by Nimis and Bargagli (Nimis and Bargagli 1999) in their multi-specific interpretative scales (Supplementary Table S3).

When inter-specific differences were tested on median values of the BEC dataset (Sect. 2.2), significant differences were highlighted for 9 out of 10 elements ($p < 0.05$), with the exception of Hg, characterized by very low values in both species (Table 3). *F. caperata* exhibited higher concentrations of As, Cd, Cu, Pb, and Zn, whereas *X. parietina* had more Al, Cr, Ni, and Ti.

Table 3. Review-based BECs (μg g^{-1} DW) for the epiphytic *Flavoparmelia caperata* and *Xanthoria parietina* in Italy. Descriptive statistics refer to the data counts (n), mean and associated standard deviation (Mean ± SD), and median and median absolute deviation (Med ± MAD) for 11 (*F. caperata*) and 10 elements (*X. parietina*) included in the BEC dataset (Sect. 2.2). Results of statistical testing (Mann–Whitney U test for independent samples) for differences between median element concentration in the two species are also reported. Significant *p*-values are highlighted in italic (n.a., data not available).

Element	*Flavoparmelia caperata*				*Xanthoria parietina*				Mann–Whitney U test		
	BEC	BEC dataset			BEC	BEC dataset					
		n	Mean ± SD	Med ± MAD		n	Mean ± SD	Med ± MAD	U	Z	*p*-value
Al	253	61	201 ± 37	209 ± 26	372	17	295 ± 59	300 ± 34	87.5	*-5.215*	*$1.8 \cdot 10^{-7}$*
As	0.18	91	0.14 ± 0.03	0.15 ± 0.02	0.15	19	0.11 ± 0.02	0.11 ± 0.01	329.0	*4.238*	*$2.3 \cdot 10^{-5}$*
Cd	0.18	68	0.14 ± 0.03	0.13 ± 0.02	0.07	19	0.05 ± 0.01	0.05 ± 0.01	13.0	*6.505*	*$7.8 \cdot 10^{-11}$*
Cr	1.17	80	0.85 ± 0.24	0.90 ± 0.20	1.61	19	1.20 ± 0.28	1.10 ± 0.20	293.0	*-4.149*	*$3.3 \cdot 10^{-5}$*
Cu	6.2	80	5.2 ± 0.9	5.5 ± 0.5	4.5	25	4.1 ± 0.3	4.1 ± 0.2	221.0	*5.859*	*$4.7 \cdot 10^{-9}$*
Hg	0.057	45	0.031 ± 0.021	0.038 ± 0.019	0.035	18	0.019 ± 0.009	0.021 ± 0.008	328.0	1.308	0.191
Ni	1.27	73	0.91 ± 0.20	0.93 ± 0.17	1.64	13	1.28 ± 0.22	1.33 ± 0.16	108.5	*-4.408*	*$1.0 \cdot 10^{-5}$*
Pb	2.37	80	1.71 ± 0.45	1.82 ± 0.48	1.00	24	0.67 ± 0.21	0.70 ± 0.21	21.0	*7.243*	*$4.4 \cdot 10^{-13}$*
Ti	19.5	46	12.8 ± 5.8	14.8 ± 4.3	37.3	11	29.3 ± 7.3	31.6 ± 4.2	22.5	*-4.652*	*$3.3 \cdot 10^{-6}$*
V	0.75	37	0.61 ± 0.11	0.62 ± 0.07	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Zn	35.3	80	29.6 ± 4.3	30.1 ± 3.2	21.3	25	17.9 ± 2.6	19.0 ± 1.9	30.0	*7.296*	*$3.0 \cdot 10^{-13}$*

Our findings were in agreement with those of Nimis et al. (2001). Indeed, these authors highlighted higher Cd and Zn in *F. caperata* than in *X. parietina* and an opposite pattern for Al and Fe (Nimis et al. 2001). Limited to Cd, Zn, and Al (Fe was excluded from our analyses), such a pattern fully matched our results, both when inter-specific differences were statistically tested in the BEC dataset (*cf.* the outcome of non-parametric statistical testing carried out for BECs in Table 3) and in the entire dataset *N* (Table 2).

The review-based BECs for *F. caperata* and *X. parietina* were generally comparable in terms of order of magnitude to those previously published for other lichens. Nevertheless, our BEC values were often lower than review-based BECs reported for pooled foliose lichen species (Bargagli 1998) and for *Hypogymnia physodes* (Bennett 1999), interestingly with the only exception of Al and Ti for *X. parietina* (*cf.* Table 3 and Supplementary Table S2). BECs for *F. caperata* and *X. parietina* were also compared to field-assessed BECs for the fruticose lichen *Pseudevernia furfuracea* based on total acid sample digestion (Cecconi et al. 2019). Even in this case, the reference values for the two foliose species were either lower than or comparable with the lowest BECs reported by the authors (*cf.* Table 3 and Supplementary Table S2).

Such data comparisons highlighted an overall pattern of comparability between the magnitude of different species-specific sets of BECs (with few exceptions, e.g., Hg). With respect to *P. furfuracea*, the lower BECs of *F. caperata* and *X. parietina* may reflect both different approaches (review-based vs. field-based BEC assessment) and lichen morphology (Incerti et al. 2017). By contrast, the higher review-based BECs reported by Bargagli are plausibly the result of aged source data, which likely included methodologically inconsistent records and bioaccumulation data from improperly defined background contexts. In this light, the assessment of review-based BECs for biological matrices should be regarded as an accurate and dynamic process, providing for the collection of methodologically uniform data for single species and involving periodical adjustments aimed at including the most recent data.

3.1.2. Bioaccumulation scale for native lichens

When bioaccumulation data in the dataset *N* were divided by the corresponding BECs (*B* ratio dataset; Data S1), inter-specific differences became negligible. Indeed, the non-parametric testing (Supplementary Methods S1) did not highlight significant *B* ratio differences between the two species (Mann–Whitney U test, $p > 0.05$; Supplementary Table S1; Supplementary Fig. S2). Therefore, the simple operation of dividing element concentration data by matched BECs had useful effects for interpretative purposes. As element concentration data and BECs are element- and species-specific, such specificity resulted flattened in the *B* ratio, allowing to develop a unique scale based on a high samples size (n = 3773).

The distribution of *B* ratios (Fig. 1) was unimodal, right-skewed, and strongly leptokurtic (skewness > 0, kurtosis > 3), as previously highlighted for the distribution of bioaccumulation data in epiphytic lichens, either pooled or not (Nimis et al. 2001).

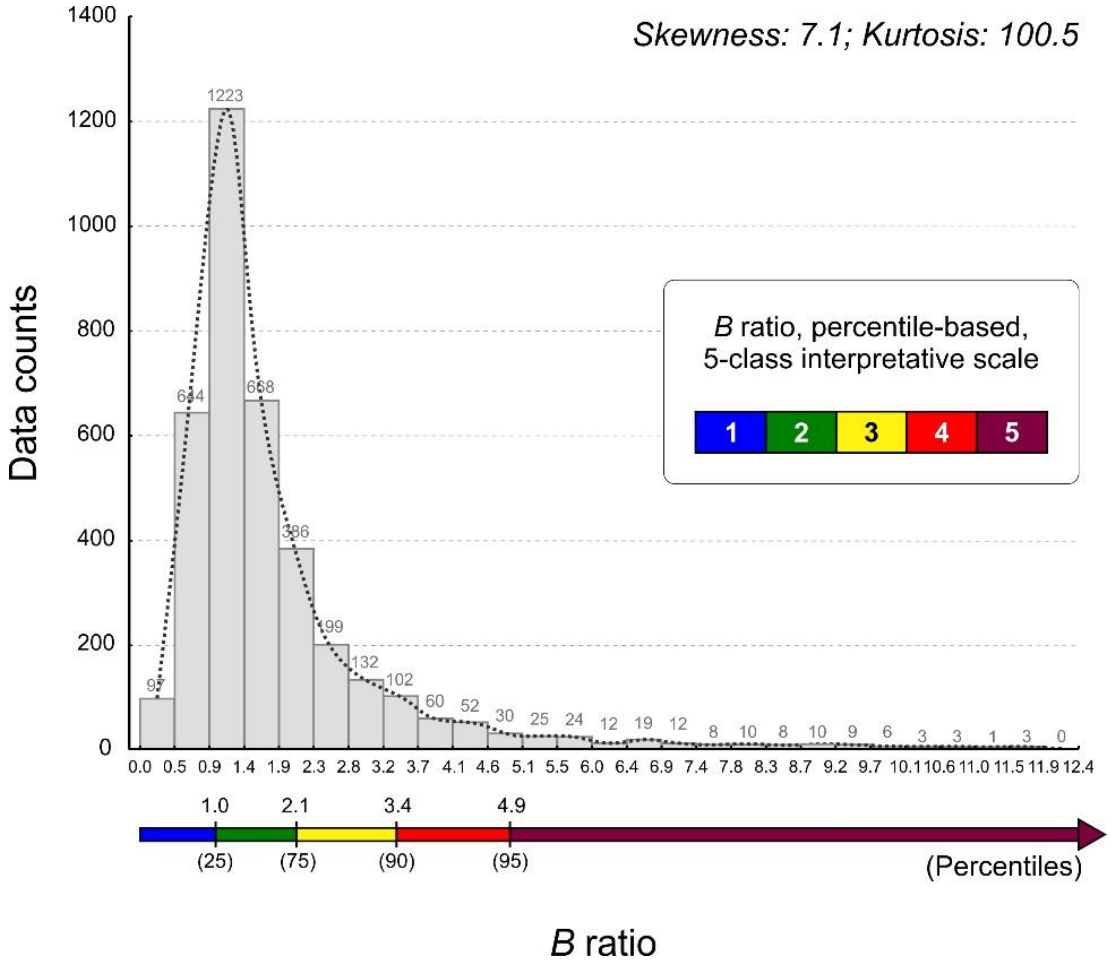


Figure 1. *B* ratio data distribution with indication of data counts per interval, skewness and kurtosis, and percentile values corresponding to the thresholds defining five bioaccumulation classes (the *B* ratio axis ends at the first 'zero' count).

The percentile thresholds for the elaboration of the new interpretative scale were redefined with respect to those of Nimis and Bargagli (Nimis and Bargagli 1999) after the appraisal of *B* ratio distributional shape. The 50th percentile was discarded because the corresponding *B* ratio value (*B* = 1.4) was too close to the BEC threshold of 1.0. The selection of the 90th percentile as upper/lower threshold of Class 3/4 (Fig. 1, Table 4) was based on toxicological considerations (Gaudet et al. 2001; Weltje and Sumpter 2017). In particular, the 90th percentile of concentration data was recently proposed as "environmentally relevant" (Weltje and Sumpter 2017), thus well suited as cutoff between the occurrence of "Moderate" and "High" bioaccumulation. Finally, the 95th percentile was chosen, instead of 98th, as the upper/lower threshold of Class 4/5 based on a precautionary approach. On these grounds, the ranges of the *B* ratio classes were characterized by similar amplitudes (Table 4).

The interpretative bioaccumulation scale was definitely improved with respect to previous multi-specific naturality/alteration scales. Indeed, besides being based on the most recent and methodologically consistent data, the *B* ratio scale is also readily understandable and provides easy implementation. As with species-specific BECs, the *B* ratio-based interpretative scale will need to be updated with the most recent data. We estimate that this might occur approximately every ten years.

Table 4. *B* ratio, percentile-based, five-class interpretative scale for bioaccumulation data from native lichens. Class codes, description and abbreviations, percentile thresholds, corresponding *B* ratio values, RGB and HTML color codes associated to bioaccumulation classes are reported.

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

The terminological shift from the previous "naturality/alteration" (Supplementary Table S3) to the more cautious "bioaccumulation level" (Table 4) may apparently pose some issues. Indeed, the latter form suggests a mere assessment of the magnitude of bioaccumulation levels in lichens, whereas the former stresses the link between lichen bioaccumulation and pollution, expressly indicating the use of scales to "assess environmental alteration in terms of a deviation from natural backgrounds" (Bargagli and Nimis 2002) (i.e., "naturality"). Yet, despite the inspiring terminology, previous scales did not rely on any operational definition of quantitative threshold for "naturality" (e.g., proper background reference), instead being based on a circular definition of "alteration" with respect to "naturality" (and vice versa) (Loppi et al. 2002). By contrast, a statistically-based element concentration benchmark (i.e., review-based BECs) is inherent to the *B* ratio, thus the new scale is actually able to assess whether or not deviations from a national unaltered reference occurred. In this light, the terminological shift was contextually driven by (*i*) the need to underline the novelty of the *B* ratio scale and (*ii*) a harmonization intent with the new scale provided for lichen transplants (see infra).

3.2. Lichen transplants

3.2.1. Source data

Before the data cleaning (Sect. 2.1), the overall *EU* ratio dataset included 820 bioaccumulation data from lichen transplant studies published over the last 25 years. Element concentration data referred to 15 elements (Al, As, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Ni, Pb, V, and Zn) analyzed in the context of 18 studies. Data referred to samples of two fruticose species, *Evernia prunastri* and *Pseudevernia furfuracea*, collected in 10 Italian administrative regions (Calabria, Campania, Emilia Romagna, Friuli Venezia Giulia, Lazio, Liguria, Lombardia, Piemonte, Toscana, Veneto). Fruticose species are usually preferred over foliose species for lichen transplants (Brunialti and Frati 2014) because they ensure greater biomass per lichen thallus, as well as easier cleaning and installation, thus contextually reducing processing time and enhancing sample homogeneity (Wolterbeek and Bode 1995). Overall, *E. prunastri* and *P. furfuracea* accounted for 18.3% and 81.7%, respectively, of data. All elements, except for Mg, included data from both lichen species.

The transplant exposure time span varied across studies: 21%, 8%, 14%, 20%, 1%, and 36% of data relied on 4, 6, 8, 9, 11, and 12 week transplants, respectively. Data relying on comparable exposure periods (i.e., 6, 8, and 9 weeks, as well as 11 and 12 weeks) were labelled as 8-week

transplant and 12-week transplant, respectively, in order to obtain three numerically balanced sub-datasets for equally spaced exposure periods. Outliers identification led to the removal of 5, 17, and 11 *EU* values from the three sub-datasets (4, 8, and 12 weeks, respectively). Eventually, the 4-week *EU* ratio sub-dataset accounted for 21% of data (n = 169 records), the 8-week sub-dataset for 42% (n = 330), and the 12-week sub-dataset for 37% (n = 288) (Data S2-S4).

3.2.2. Bioaccumulation Scale for Lichen Transplants

Even in the case of bioaccumulation data from lichen transplants, inter-specific differences were negligible when addressed by non-parametric testing on *EU* ratios. Indeed, the output of statistical testing (Supplementary Methods S1) did not highlight significant *EU* ratio differences between the two species (Mann–Whitney U test, $p > 0.05$; Supplementary Table S1; Supplementary Fig. S3), thus the same considerations spelt out for *B* ratios apply. *EU* ratio distributions were right-skewed and slightly platykurtic (skewness > 0, kurtosis < 3; Fig. 2). The positive skewness was consistent (although lower) with that of the *B* ratio distribution.

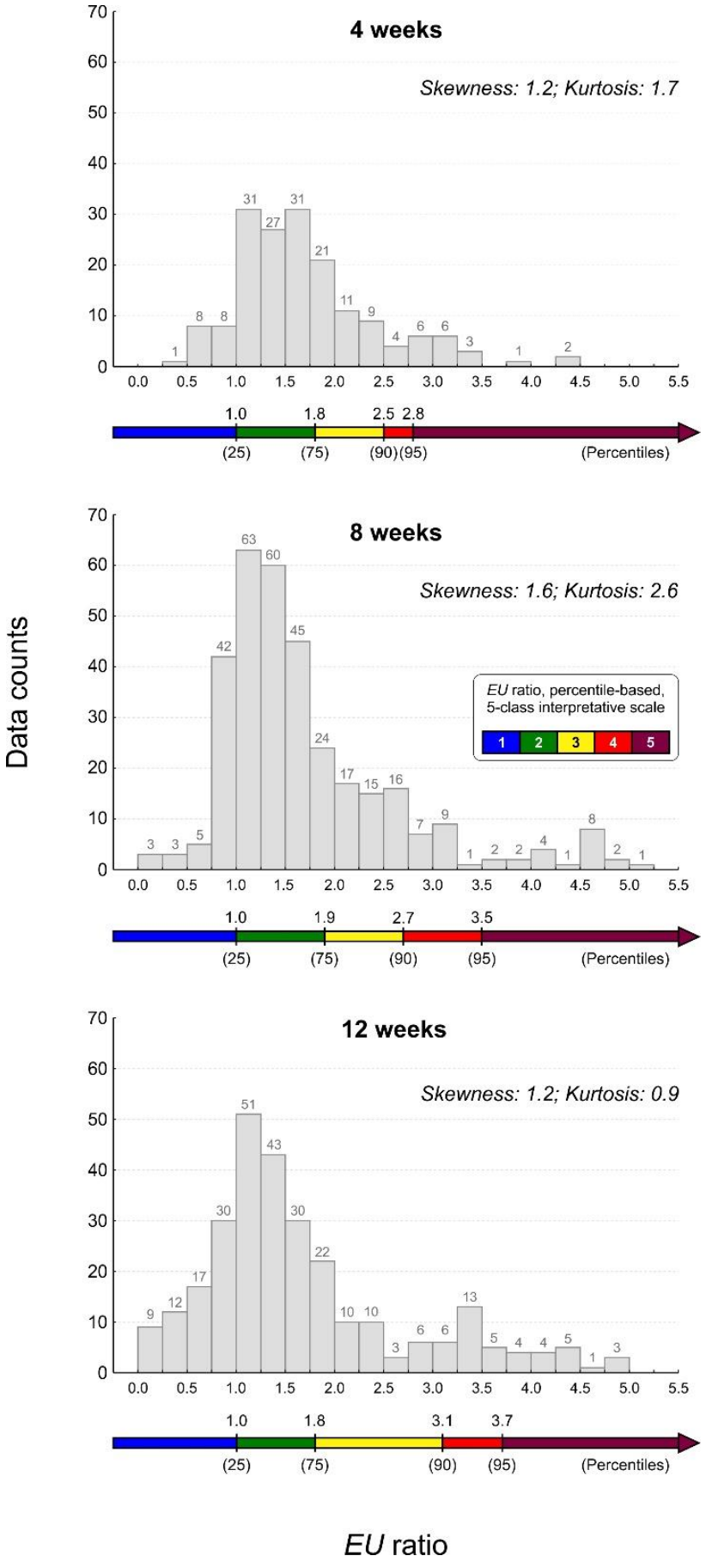


Figure 2. *EU* ratio data distribution with indication of data counts per interval, skewness and kurtosis, and percentile values corresponding to the thresholds defining five bioaccumulation classes. Data are separately reported for different transplant exposure time spans (from the top to the bottom: 4, 8, and 12 weeks).

Certainly, the marked differences between the distributions of *B* and *EU* ratios were because of different (*i*) sample sizes (Sect. 3.1.2 and 3.2.1), (*ii*) benchmark values in the ratio denominators (i.e., BECs vs. elemental concentration values of unexposed samples), and (*iii*) duration of lichen exposure to pollutants and bioaccumulation mechanisms. Concerning the latter point, transplanted lichens are exposed for few weeks to new, and often harsh, environmental conditions, thus rapidly accumulating mostly through passive mechanisms (Nieboer et al. 1978; Nash 2008). By contrast, the bioaccumulation in lifespan-exposed native lichens is the result of a long-term interplay of both passive phenomena and slower active intracellular uptakes characterized by element-specific kinetics (Brown and Beckett 1984). Such interplay eventually results in the achievement of a dynamic equilibrium with the surrounding environment (Paoli et al. 2018) and likely in higher elemental concentration levels in the case of important pollutant loads. Indeed, *EU* ratios corresponding to 90th and 95th percentiles of the distributions were lower than the corresponding *B* ratio values (*cf.* Table 4 and Table 5).

In transplants, the importance of the exposure time span (Gailey and Lloyd 1986; Mikhailova 2002) emerged in the *EU* ratio data in the case of high pollutant loads. Indeed, an increasing trend of *EU* ratio values corresponding to 90th and 95th percentiles was observed when moving from 4 to 12 week exposure (Fig. 2, Table 5). Utmost differences were highlighted for the *EU* ratio corresponding to the 95th percentile between the 4 week exposure and 8 or 12 week exposures (2.8 vs. 3.5 and 3.7; Table 5). Interestingly, no trend of increasing values from 4 to 12 weeks was highlighted for *EU* ratio values corresponding to 25th and 75th percentiles, confirming that the exposure time span mostly affects bioaccumulation results in the case of high levels of airborne pollutant depositions (*EU* ratio above 90th percentile, i.e., environmentally relevant bioaccumulation (Weltje and Sumpter 2017). On this basis, three different series of values have been reported, to be alternatively used according to the selected exposure time span. Nevertheless, it should also be pointed out that in most biomonitoring literature targeting mosses, short exposure times (i.e., 3–4 weeks) are discouraged because unclear "accumulation signals" would lead to the construction of derived datasets of limited reliability (Capozzi et al. 2016). Such a methodological issue has been dealt more rarely for lichen transplants, but it is generally agreed that lichens should be exposed for at least 6–8 weeks, based on the following considerations: detectable accumulated concentrations, replicability, and exposure time spans within the limits of practical considerations (Gailey and Lloyd 1986).

Table 5. *EU* ratio, percentile-based, five-class interpretative scale for bioaccumulation data from lichen transplants. Class codes, description and abbreviations, percentile thresholds, corresponding *EU* ratio values for different exposure time spans, and color codes (RGB and HTML) associated with bioaccumulation classes are reported.

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

* The *EU* ratio values corresponding to 25th percentile threshold (upper/lower threshold of Class 1/2) are actually equal to 1.1, 1.0 and 0.9 for exposure time spans of 4, 8 and 12 weeks, respectively (see text for explanation).

In the bioaccumulation scale proposed for lichen transplants, the upper/lower *EU* ratio threshold of Class 1/2 was aprioristically established at *EU* = 1 (Sect. 2.3). However, it is worth noting that the corrected *EU* ratio values corresponding to the 25th percentile are actually very close to such a value (ranging from 0.9 to 1.1, see footnote in Table 5; Data S2–S4).

Even in this case, we decided to abandon the old class description based on the concept of "loss", because an element concentration decrease may either reflect actual "pristine" ambient air conditions at the transplant sites or a "washing effect" caused by rainfall in the presence of non-negligible pollutant emissions (Bargagli and Mikhailova 2002). Finally, it must be pointed out that, given the relatively limited amount of source data, this new bioaccumulation scale for lichen transplants has to be regarded as preliminary and should be used with caution, pending the inclusion of new available bioaccumulation data.

3.3 Comparison between previous and new interpretative scales

The naturality/alteration scales applied in the last twenty years in Italy (Nimis and Bargagli 1999) and the brand-new bioaccumulation scale for native lichens (Table 4) were both applied to the same case study. In this case, a direct comparison between classes attributed to sampling sites resulting from different interpretative scales would be pointless because of the substantial differences in scale conceptualization; however, a comparative analysis of outcomes permitted some interesting considerations.

Overall, the application of previous scales provided a rather optimistic description of the study area. According to the seven-class scale, the vast majority of sampling sites were characterized by "very high" and "high naturality". In particular, 96.5%, 89.6%, and 86.2% of sites belonged to such classes for As, Cd, and Cr concentration in *Flavoparmelia caperata*, as well as 88.9% (As and Cd) and 77.7% (Cr) for *Xanthoria parietina* (Fig. 3, Supplementary Table S5). "Low alteration" characterized only two sites for Cr (G6 and D7 for *F. caperata* and *X. parietina*, respectively; Fig. 3).

When the new bioaccumulation scale was applied, the majority of sampling sites were consistently characterized by "Low bioaccumulation", with the exception of As in *X. parietina*, instead characterized by a majority of sites belonging to Class 3 ("Moderate bioaccumulation"). In particular, 69.0%, 79.3%, and 82.8% belonged to Classes 1 and 2 for As, Cd, and Cr concentration, respectively, in *F. caperata*, as well as 33.3% (As), 66.7% (Cd), and 88.9% (Cr) for *X. parietina*. However, by applying this scale, some cases of "High" and "Severe bioaccumulation" were also highlighted (Fig. 3, Supplementary Table S5), thus determining a more conservative interpretation.

The study area is characterized by high anthropogenic pressure and the presence of a coal-fired thermoelectric power plant, shipbuilding industries, and other small industrial activities (ARPA FVG). Previous investigations demonstrated that, overall, the elemental concentrations in lichens grown in the study area were not impressively high; however, a certain contamination of As and Cr occurred. These elements are acknowledged tracers of coal combustion (Zhang et al. 2007; Wang et al. 2018); therefore, the enrichment observed in thalli collected at specific sites was ascribed to the power plant emissions (ARPA FVG), although these were compliant with threshold limits (ARIANET). In particular, the evidence that Cr concentration in lichen samples was related to the

plant emissions was confirmed by the results of an air particulate matter sampling carried out during both operational and non-operational state of the plant (Fortuna 2018).

The pattern revealed by the new scale correlates well with the deposition plume highlighted by traditional modelling approaches, particularly for As. Indeed, the deposition plume starts from the power plant (E6) and develops over the east–west axis following the prevailing wind direction blowing from the east (as modulated by the local orography) (ARPA FVG; Fortuna 2018). By contrast, the application of previous scales failed to represent the actual variations in element depositions affecting the whole area, especially for As. Regarding Cd, it should be preliminary stated that the rather low recovery (70%, Sect. 2.3) could introduce a certain bias in element content results and cartographic output. Having said that, previous and new scales identified a consistent pattern for sites located in the proximity of shipbuilding activities (i.e., C6-7, D6-7, E6, F6, G6), but again, previous scales provided a certainly more optimistic scenario (*cf.* sites D7 and G6; Fig. 3).

The reasons for the general worse performance of the naturality/alteration scales have to be sought in the source dataset. Indeed, this included rather old studies often reporting high element concentration values, which consequently affected data distributions and resulted in a general underestimation of pollutant depositions (*cf.* median values of Table 2 with values corresponding to 50^{th} percentiles in Supplementary Table S3), thus explaining the misleading outcome obtained for As. This is further evidence that interpretative scales obtained through a meta-analytical approach may quickly become obsolete as a result of rapidly changing scenarios, for example, variations in pollutant emissions determined by a plethora of anthropogenic and non-anthropogenic causes (i.e., abatement or increasing traffic-related pollution, introduction of environmental protection measures, long-range atmospheric transport, and so on (Wilson and Horrocks 2008; Madsen et al. 2011; Kollanus et al. 2016).

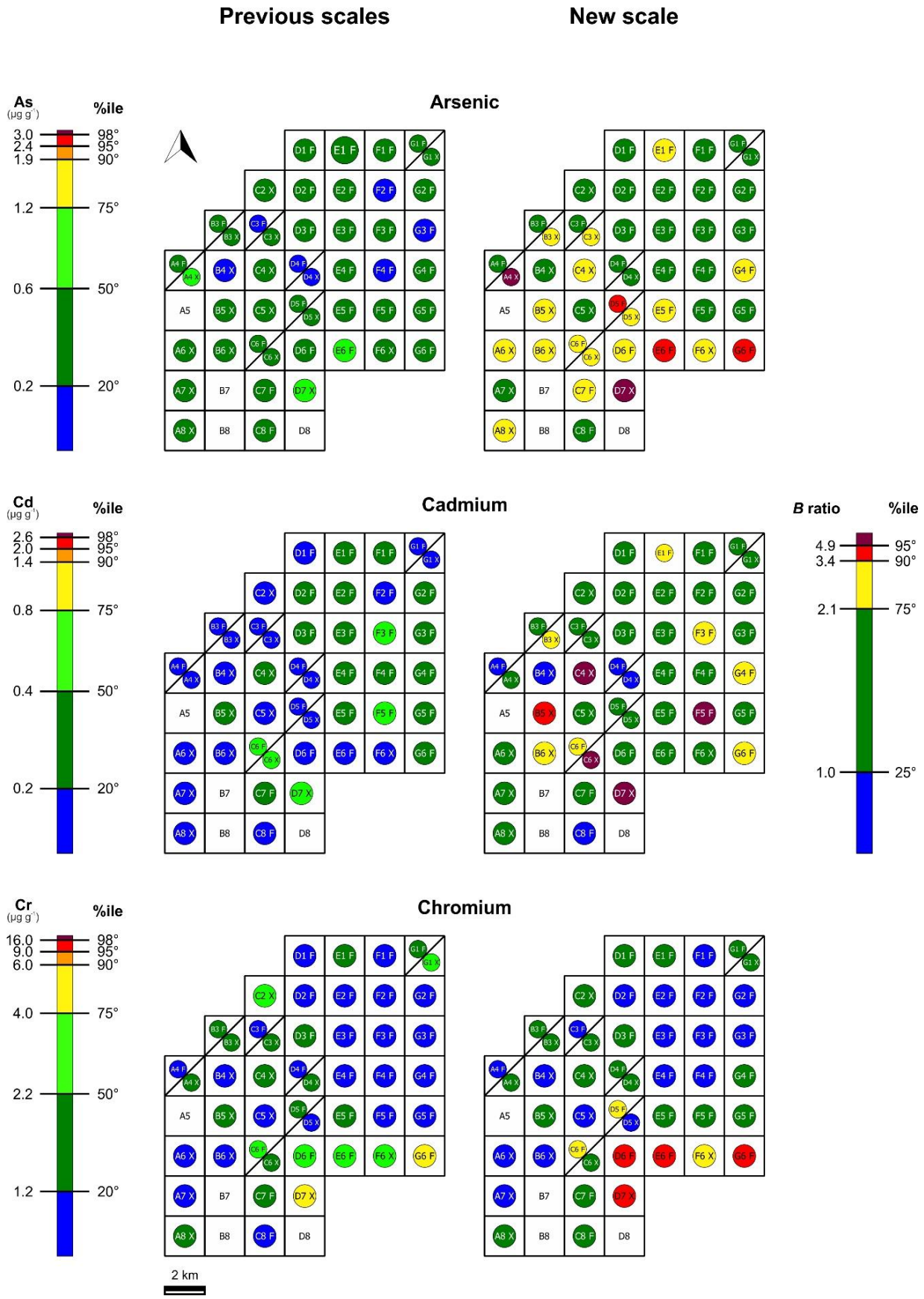


Figure 3. Cartographic representation of sampling sites and corresponding classes of the naturality/alteration scale (Nimis and Bargagli 1999) (here, "previous scales") and bioaccumulation scale (Table 4; here, "new scale"), with indication of percentile thresholds (%ile), corresponding element concentration values (left), and *B* ratios (right). Sampling sites are identified by alphanumeric codes (as also reported in Supplementary Table S5) followed by the letter F or X for *Flavoparmelia caperata* and *Xanthoria parietina*, respectively.

The accumulation/loss scale (Frati et al. 2005) and the brand-new bioaccumulation scale for lichen transplants (Table 5) were both applied to the same case study. The concentration of As, Cd, and Cr significantly increased in the samples of *Pseudevernia furfuracea* after 12-week exposure, although enrichment levels were not indicative of strong contamination (Tretiach et al. 2011).

According to the previous scale (Frati et al. 2005), transplant sites were characterized by "normal" accumulation (Supplementary Table S6) in 66.7%, 46.7%, and 40% of cases for As, Cd, and Cr, respectively. Instead, "accumulation" or "severe accumulation" occurred in 33.3% (As), 53.3% (Cd), and 60.0% (Cr) of cases. When the new scale was applied, the great majority of sites were characterized by "Absence of bioaccumulation" or "Low bioaccumulation"; in particular, 96.7% (As), 86.7% (Cd), and 90.0% (Cr). "Severe bioaccumulation" was highlighted in samples exposed in a single site (3.3%) limited to As, whereas "Moderate bioaccumulation" characterized 13.3% (Cd) and 10.0% (Cr) of sites.

The main limitations of the accumulation/loss scale are evident. Indeed, its use determines (*i*) a heavy flattening of element concentration variations concerning enrichments exceeding 75% (which are uncompromisingly identified as "severe accumulation"), and (*ii*) an exacerbation of slighter variations (i.e., enrichments between 24% and 76%, which are considered to range between "normal" and "severe" accumulation"). A case in point is represented by the highest values revealed for As in the study area: indeed, the two highest exposed values were 0.37 µg g^{-1} (*EU* = 1.50) and 2.34 µg g^{-1} (*EU* = 9.35), measured in samples exposed in D5 and B2, respectively (Fig. 4; Supplementary Table S6). Using the accumulation/loss scale, the difference between an increase of 50% (D5) and an increase of 835% (B2) with respect to the unexposed levels is poorly reflected by a single class step (from "accumulation" to "severe accumulation"; Supplementary Table S4). By contrast, such a large difference is far better reflected by the three class steps of the bioaccumulation scale (from "Low" to "Severe" bioaccumulation; Table 5).

Another issue inherent to the use of the previous scale concerns the precision achieved in determining mean element concentration values of unexposed samples (i.e., the closeness of agreement among the set of element concentration results; Gotelli and Ellison 2012). A proper assessment of such a reference value may indeed be a non-trivial task. Operators usually average element concentration values measured in a certain number of samples taken from thalli randomly selected from bulked lichen material. Obviously, this should be based on adequate sample size, which in turn should be established on the basis of a preliminary characterization of the elemental concentration variability of the target lichen matrix in the background site. However, the mean value of unexposed samples is often assessed by analyzing too few samples (frequently n = 3), and this could have potential interpretative consequences when using a scale based on classes of limited width such as the accumulation/loss scale (Frati et al. 2005). As a matter of fact, when element concentration values are few and highly dispersed (e.g., coefficient of variation > 1), and especially in case of rather low enrichments, the ascription of the *EU* value to a bioaccumulation class may result in a pointless procedure, as these conditions would not guarantee repeatability.

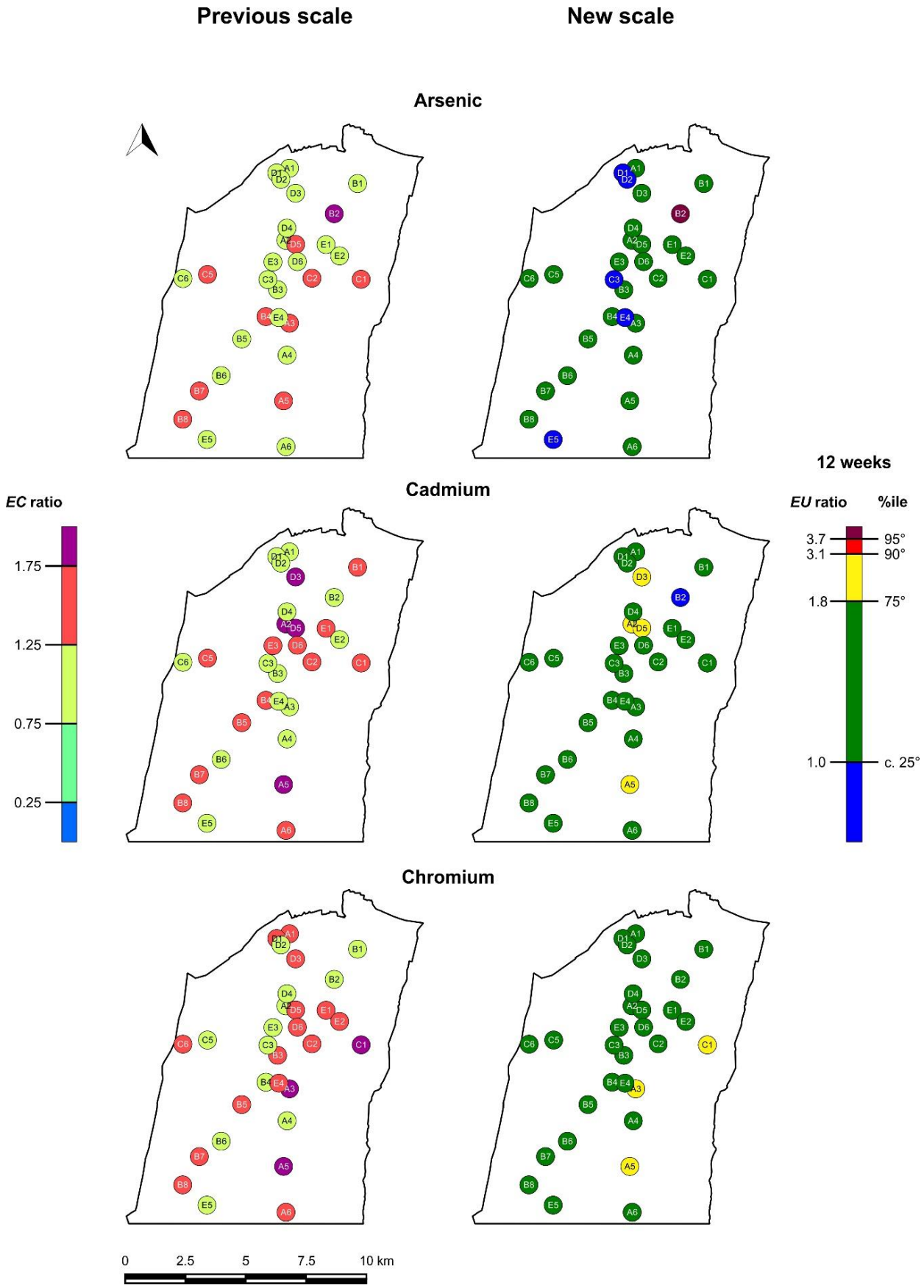


Figure 4. Cartographic representation of *Pseudevernia furfuracea* transplant sites, corresponding classes of the accumulation/loss scale (Frati et al. 2005) (here, "previous scale") and the bioaccumulation scale (Table 5; here, "new scale"), with indication of *EC* ratios (Table 1) for the former, percentile thresholds (%ile), and *EU* ratios for the latter. Transplant sites are identified by alphanumeric codes (as also reported in Supplementary Table S6).

4. Conclusions

In biomonitoring, interpretative scales are fundamental to the assessment of the magnitude of pollution phenomena. Until now, scales based on very different assumptions have been developed: the so-called "naturality/alteration scales", for biomonitoring with native lichens; and the "accumulation/loss scale", for transplant-based applications. Despite their popular use in Italy and abroad, both scales were never critically reappraised, notwithstanding some evident methodological flaws.

By recovering some core ideas from previous scales, we developed new interpretative scales based on the meta-analysis of methodologically consistent bioaccumulation data from the most recent Italian literature. The distributions of the ratios between element concentration data and species-specific background (*B* ratio, native lichens) or element concentration of unexposed samples (*EU* ratio, transplants) were analyzed. On this basis, two easily enforceable, percentile-based, five-class "Bioaccumulation scales" were set up. A critical revision of scale-associated terminology was also proposed. For both native lichens and transplants, the five classes refer to (1) "Absence of bioaccumulation" (A), (2) "Low bioaccumulation" (L), (3) "Moderate bioaccumulation" (M), (4) "High bioaccumulation" (H), and (5) "Severe bioaccumulation" (S), with *B* and *EU* ratio thresholds corresponding to the 25^{th}, 75^{th}, 90^{th}, and 95^{th} percentiles of their distributions.

The comparative application of previous and new scales to two case studies suggested a better and more consistent performance of the latter. Moreover, it also demonstrated that scales developed on the basis of real biomonitoring data may become obsolete owing to changing scenarios, thereby leading to the need for periodical updating with the inclusion of new available data to the source datasets.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS S1

In order to assess whether *B* and *EU* ratios were substantially unaffected by inter-specific differences, their median values were tested for significant differences using Mann-Whitney's U test for independent samples. Median *B* ratios were tested using the column vector reported in Data S1. Median *EU* ratios were tested before outlier removal (overall *EU* ratio data), and separately in each *EU ratio* sub-dataset after outlier removal (exposure time spans of 4, 8 and 12 weeks; Data S2-S4). Statistical significance was tested in all cases at $\alpha = 0.05$ (Supplementary Table S1).

SUPPLEMENTARY TABLES S1-S6

Supplementary Table S1. Results of statistical testing (Mann-Whitney U test for independent samples) for differences between median values of *B* ratios (native lichens) in *Flavoparmelia caperata* and *Xanthoria parietina*, as well as between median values of *EU* ratios (lichen transplants) in *Evernia prunastri* (*Ep*) and *Pseudevernia furfuracea* (*Pf*) (either reported for the overall data or separately for each exposure time span). Data counts (n) are reported in brackets in the headers.

***B* ratio** (*Flavoparmelia caperata*, n = 3005; *Xanthoria parietina*, n = 768)		
U	**Z**	***p*-value**
1˙120˙389	1.245	0.213

***EU* ratio** (*Evernia prunastri*, n = 150; *Pseudevernia furfuracea*, n = 670)			
Exposure time span	**U**	**Z**	***p*-value**
Overall	49˙992	0.098	0.922
4 weeks (*Ep*, n = 77; *Pf*, n = 92)	3406	-0.428	0.669
8 weeks (*Ep*, n = 24; *Pf*, n = 306)	3164	1.128	0.259
12 weeks (*Ep*, n = 48; *Pf*, n = 240)	5406	0.671	0.502

Supplementary Table S2. Lichen Background Element Concentration values (BECs, µg g^{-1} DW) reported by Bargagli (1998), Bennett (1999) and Cecconi et al. (2019), with indication of target lichen species, reference areas and number of sampling sites (in brackets, when available). Data reported by Bargagli refer to element concentration ranges (Bargagli 1998).

Element	**Bargagli** [1]	**Bennett** [1]		**Cecconi et al.** [2]			
	Foliose species	***Hypogymnia physodes***		***Pseudevernia furfuracea***			
	Multiple areas	**Multiple areas**		**Eastern Alps** (12)	**Central Alps** (13)	**Western Alps** (11)	**Apennines** (18)
Al	150 ÷ 300	448	(32)	688	1024	835	1600
As	0.7 ÷ 2.0	1.33	(7)	0.611	0.605	0.398	0.407
Cd	< 0.1 ÷ 0.3	0.562	(37)	0.159	0.204	0.155	0.251
Cr	1 ÷ 4	2.11	(33)	5.26	5.93	7.02	8.33
Cu	4 ÷ 10	5.96	(37)	4.5	7.36	4.79	4.95
Hg	< 0.1 ÷ 0.2	0.253	(15)	-	-	-	-
Ni	1 ÷ 3	1.72	(33)	1.17	1.83	4.46	1.77
Pb	1 ÷ 8	19.5	(39)	2.77	4.49	2.35	4.6
Ti	5 ÷ 35	26.4	(5)	43.4	60.2	55	106
V	< 1 ÷ 3	16.9	(8)	1.2	1.88	1.41	2.8
Zn	20 ÷ 90	73	(43)	38	64.4	40	31.4

[1] Review-based BECs; [2] Field-assessed BECs.

Supplementary Table S3. Percentile-based, 7-class, naturality/alteration scale for bioaccumulation data provided by Nimis and Bargagli (1999) (the abbreviations in brackets are the same used in Supplementary Table S5). Data refer to percentile thresholds, corresponding element concentration values (µg g^{-1} DW) for the 11 elements included in the *N* dataset after the methodological data filtering (Sect. 2.2 and 3.1), and the colours suggested by the authors (Nimis and Bargagli 1999) (data counts are reported in brackets below each element).

	Class (abbreviation)		**Percentile**	**Al** (626)	**As** (435)	**Cd** (626)	**Cr** (654)	**Cu** (656)	**Hg** (606)	**Ni** (655)	**Pb** (699)	**Ti** (138)	**V** (416)	**Zn** (699)	**Color**
1	Very high naturality	(V.h.n)	< 20^{th}	< 350	< 0.2	< 0.2	< 1.2	< 7	< 0.07	< 1	< 4	< 13	< 0.63	< 30	Blue
2	High naturality	(H.n.)	20^{th} - 50^{th}	350-600	0.2-0.6	0.2-0.4	1.2-2.2	7-10	0.07-0.13	1-2	4-10	13-27	0.63-1.7	30-40	Dark green
3	Middle naturality	(M.n.)	50^{th} - 75^{th}	600-1000	0.6-1.2	0.4-0.8	2.2–4.0	10-15	0.13-0.20	2-3	10-25	27-70	1.7-3.1	40-65	Pale green
4	Low nat./alteration	(L.a.)	75^{th} - 90^{th}	1000-1600	1.2-1.9	0.8-1.4	4.0–6.0	15-25	0.20-0.29	3-5	25-55	70-97	3.1-5.1	65-94	Yellow
5	Middle alteration	(M.a.)	90^{th} - 95^{th}	1600-2500	1.9-2.4	1.4–2.0	6.0–9.0	25-34	0.29-0.42	5-6	55-80	97-113	5.1-6.7	94-115	Orange
6	High alteration	(H.a.)	95^{th} - 98^{th}	2500-3200	2.4–3.0	2.0-2.6	9.0–16.0	34-53	0.42-0.74	6-8	80-108	113-150	6.7-9.3	115-155	Red
7	Very high alteration	(V.h.a.)	> 98^{th}	> 3200	> 3.0	> 2.6	>16.0	> 53	0.74	> 8	> 108	> 150	> 9.3	> 155	Crimson

Supplementary Table S4. Accumulation/loss scale provided by Frati et al. (2005) with 5 *EC* ratio classes (the abbreviations in brackets are the same used in Supplementary Table S6).

Accumulation/loss (abbreviation)		***EC* ratio**
Severe loss	(S.l.)	0 – 0.25
Loss	(Loss)	0.25 – 0.75
Normal	(N.)	0.75 – 1.25
Accumulation	(Acc.)	1.25 – 1.75
Severe accumulation	(S.a.)	> 1.75

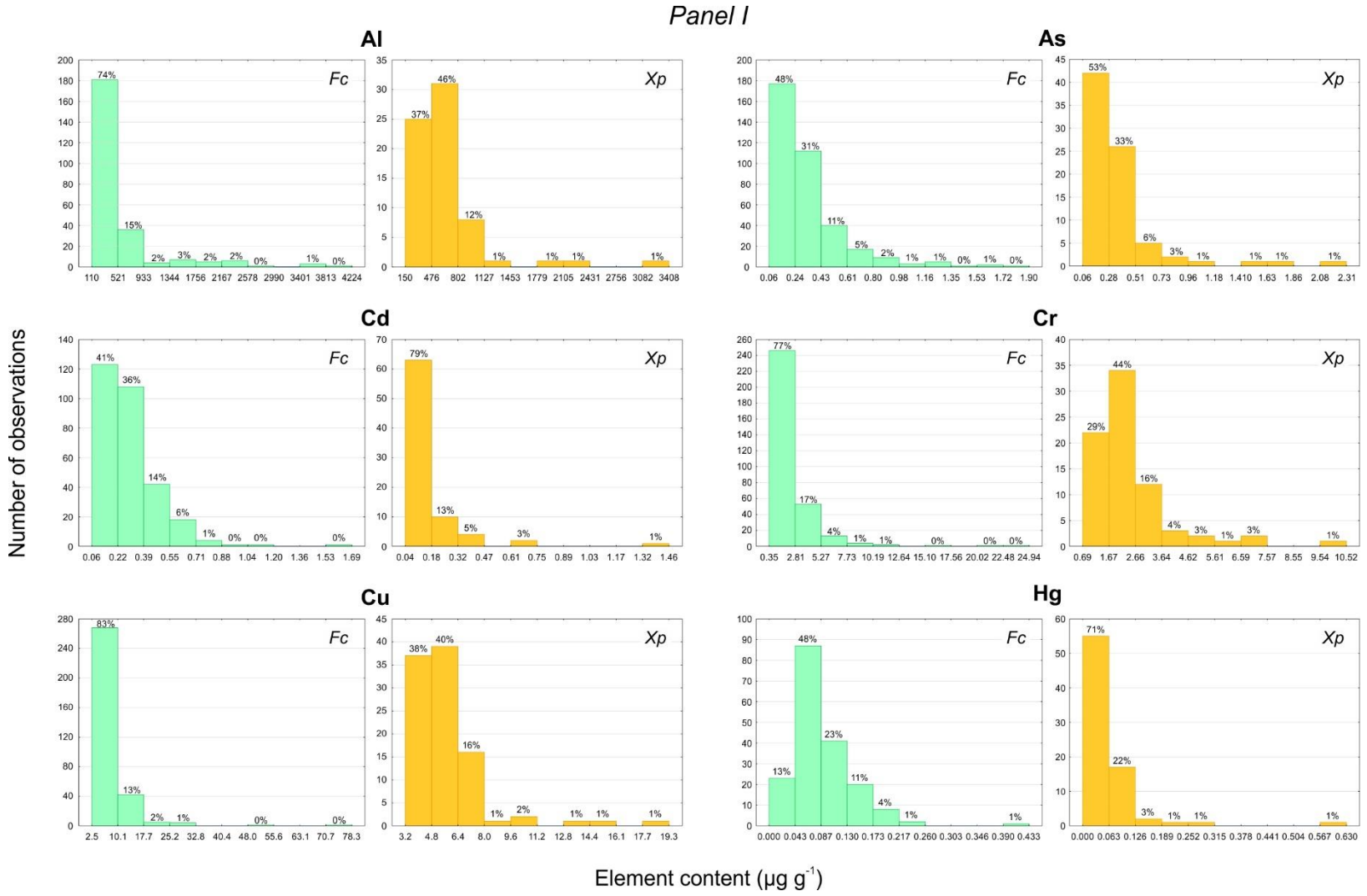
Supplementary Table S5. Results of the comparative application of the naturality/alteration scales by Nimis and Bargagli (1999) (nat./alt. class) and the bioaccumulation scale in Table 4 (B. class) to a real case study. Data refer to three elements (As, Cd, Cr) measured in samples of *Flavoparmelia caperata* and *Xanthoria parietina* collected in 40 sampling sites (Fig. 3). Abbreviations for classes of naturality/alteration and bioaccumulation scales are reported in Supplementary Table S3 and Table 4. The attribution of classes is based on absolute element concentration expressed in µg g^{-1} DW (Elem. conc.) for naturality/alteration scales, whereas it is based on the *B* ratio for the bioaccumulation scale.

Site	*Flavoparmelia caperata*												*Xanthoria parietina*											
	As				Cd				Cr				As				Cd				Cr			
	Elem. conc.	Nat./alt. class	*B* ratio	B. class	Elem. conc.	Nat./alt. class	*B* ratio	B. class	Elem. conc.	Nat./alt. class	*B* ratio	B. class	Elem. conc.	Nat./alt. class	*B* ratio	B. class	Elem. conc.	Nat./alt. class	*B* ratio	B. class	Elem. conc.	Nat./alt. class	*B* ratio	B. class
A4	0.23	2 (H.n.)	1.65	2 (L)	0.11	1 (V.h.n.)	0.81	1 (A)	0.84	1 (V.h.n.)	0.99	1 (A)	0.85	3 (M.n.)	8.12	5 (S)	0.07	1 (V.h.n.)	1.24	2 (L)	1.40	2 (H.n.)	1.17	2 (L)
A6													0.28	2 (H.n.)	2.67	3 (M)	0.07	1 (V.h.n.)	1.31	2 (L)	1.10	1 (V.h.n.)	0.92	1 (A)
A7													0.20	2 (H.n.)	1.91	2 (L)	0.09	1 (V.h.n.)	1.72	2 (L)	0.86	1 (V.h.n.)	0.72	1 (A)
A8													0.22	2 (H.n.)	2.10	3 (M)	0.10	1 (V.h.n.)	1.92	2 (L)	1.41	2 (H.n.)	1.17	2 (L)
B3	0.27	2 (H.n.)	1.94	2 (L)	0.18	1 (V.h.n.)	1.33	2 (L)	1.61	2 (H.n.)	1.91	2 (L)	0.25	2 (H.n.)	2.34	3 (M)	0.12	1 (V.h.n.)	2.21	3 (M)	1.75	2 (H.n.)	1.46	2 (L)
B4													0.12	1 (V.h.n.)	1.15	2 (L)	0.05	1 (V.h.n.)	0.87	1 (A)	1.10	1 (V.h.n.)	0.92	1 (A)
B5													0.32	2 (H.n.)	3.06	3 (M)	0.22	2 (H.n.)	4.06	4 (H)	1.30	2 (H.n.)	1.08	2 (L)
B6													0.29	2 (H.n.)	2.77	3 (M)	0.12	1 (V.h.n.)	2.22	3 (M)	0.92	1 (V.h.n.)	0.77	1 (A)
C2													0.20	2 (H.n.)	1.91	2 (L)	0.07	1 (V.h.n.)	1.22	2 (L)	2.30	3 (M.n.)	1.92	2 (L)
C3	0.18	1 (V.h.n.)	1.29	2 (L)	0.17	1 (V.h.n.)	1.23	2 (L)	0.67	1 (V.h.n.)	0.79	1 (A)	0.29	2 (H.n.)	2.72	3 (M)	0.09	1 (V.h.n.)	1.62	2 (L)	1.45	2 (H.n.)	1.21	2 (L)
C4													0.24	2 (H.n.)	2.29	3 (M)	0.28	2 (H.n.)	5.17	5 (S)	1.60	2 (H.n.)	1.34	2 (L)
C5													0.21	2 (H.n.)	2.01	2 (L)	0.09	1 (V.h.n.)	1.64	2 (L)	0.97	1 (V.h.n.)	0.81	1 (A)
C6	0.31	2 (H.n.)	2.22	3 (M)	0.45	3 (M.n.)	3.32	3 (M)	2.20	3 (M.n.)	2.60	3 (M)	0.25	2 (H.n.)	2.39	3 (M)	0.64	3 (M.n.)	11.82	5 (S)	2.00	2 (H.n.)	1.67	2 (L)
C7	0.35	2 (H.n.)	2.51	3 (M)	0.24	2 (H.n.)	1.77	2 (L)	1.50	2 (H.n.)	1.77	2 (L)												
C8	0.27	2 (H.n.)	1.94	2 (L)	0.12	1 (V.h.n.)	0.89	1 (A)	1.10	1 (V.h.n.)	1.30	2 (L)												
D1	0.20	2 (H.n.)	1.43	2 (L)	0.18	1 (V.h.n.)	1.33	2 (L)	0.92	1 (V.h.n.)	1.09	2 (L)												
D2	0.21	2 (H.n.)	1.51	2 (L)	0.27	2 (H.n.)	1.96	2 (L)	0.77	1 (V.h.n.)	0.90	1 (A)												
D3	0.28	2 (H.n.)	2.01	2 (L)	0.23	2 (H.n.)	1.66	2 (L)	1.38	2 (H.n.)	1.63	2 (L)												
D4	0.16	1 (V.h.n.)	1.15	2 (L)	0.11	1 (V.h.n.)	0.81	1 (A)	1.10	1 (V.h.n.)	1.30	2 (L)	0.18	1 (V.h.n.)	1.69	2 (L)	0.05	1 (V.h.n.)	1.00	1 (A)	1.27	2 (H.n.)	1.06	2 (L)
D5	0.51	2 (H.n.)	3.66	4 (H)	0.18	1 (V.h.n.)	1.33	2 (L)	2.10	2 (H.n.)	2.48	3 (M)	0.26	2 (H.n.)	2.48	3 (M)	0.07	1 (V.h.n.)	1.29	2 (L)	0.69	1 (V.h.n.)	0.58	1 (A)
D6	0.30	2 (H.n.)	2.15	3 (M)	0.15	1 (V.h.n.)	1.11	2 (L)	3.45	3 (M.n.)	4.08	4 (H)												
D7													1.00	3 (M.n.)	9.55	5 (S)	0.75	3 (M.n.)	13.85	5 (S)	5.10	4 (L.n.)	4.26	4 (H)
E1	0.35	2 (H.n.)	2.47	3 (M)	0.37	2 (H.n.)	2.69	3 (M)	1.45	2 (H.n.)	1.71	2 (L)												
E2	0.20	2 (H.n.)	1.40	2 (L)	0.20	2 (H.n.)	1.44	2 (L)	0.80	1 (V.h.n.)	0.94	1 (A)												
E3	0.23	2 (H.n.)	1.65	2 (L)	0.24	2 (H.n.)	1.73	2 (L)	0.71	1 (V.h.n.)	0.84	1 (A)												
E4	0.24	2 (H.n.)	1.72	2 (L)	0.24	2 (H.n.)	1.77	2 (L)	0.56	1 (V.h.n.)	0.66	1 (A)												
E5	0.33	2 (H.n.)	2.37	3 (M)	0.20	2 (H.n.)	1.48	2 (L)	1.60	2 (H.n.)	1.89	2 (L)												
E6	0.61	3 (M.n.)	4.34	4 (H)	0.19	1 (V.h.n.)	1.37	2 (L)	3.65	3 (M.n.)	4.31	4 (H)												
F1	0.21	2 (H.n.)	1.51	2 (L)	0.20	2 (H.n.)	1.48	2 (L)	0.84	1 (V.h.n.)	0.99	1 (A)												
F2	0.18	1 (V.h.n.)	1.25	2 (L)	0.19	1 (V.h.n.)	1.37	2 (L)	0.59	1 (V.h.n.)	0.69	1 (A)												
F3	0.26	2 (H.n.)	1.89	2 (L)	0.44	3 (M.n.)	3.22	3 (M)	0.57	1 (V.h.n.)	0.67	1 (A)												
F4	0.19	1 (V.h.n.)	1.33	2 (L)	0.24	2 (H.n.)	1.77	2 (L)	0.53	1 (V.h.n.)	0.62	1 (A)												
F5	0.26	2 (H.n.)	1.86	2 (L)	0.67	3 (M.n.)	4.95	5 (S)	1.00	1 (V.h.n.)	1.18	2 (L)												
F6													0.31	2 (H.n.)	2.96	3 (M)	0.10	1 (V.h.n.)	1.77	2 (L)	3.10	3 (M.n.)	2.59	3 (M)
G1	0.23	2 (H.n.)	1.65	2 (L)	0.16	1 (V.h.n.)	1.14	2 (L)	1.20	2 (H.n.)	1.42	2 (L)	0.20	2 (H.n.)	1.91	2 (L)	0.06	1 (V.h.n.)	1.16	2 (L)	2.50	3 (M.n.)	2.09	2 (L)
G2	0.21	2 (H.n.)	1.51	2 (L)	0.24	2 (H.n.)	1.75	2 (L)	0.68	1 (V.h.n.)	0.80	1 (A)												
G3	0.20	1 (V.h.n.)	1.41	2 (L)	0.27	2 (H.n.)	1.99	2 (L)	0.45	1 (V.h.n.)	0.53	1 (A)												
G4	0.31	2 (H.n.)	2.22	3 (M)	0.29	2 (H.n.)	2.10	3 (M)	1.02	1 (V.h.n.)	1.21	2 (L)												
G5	0.23	2 (H.n.)	1.62	2 (L)	0.21	2 (H.n.)	1.53	2 (L)	0.97	1 (V.h.n.)	1.15	2 (L)												
G6	0.48	2 (H.n.)	3.44	4 (H)	0.33	2 (H.n.)	2.40	3 (M)	4.10	4 (L.n.)	4.85	4 (H)												

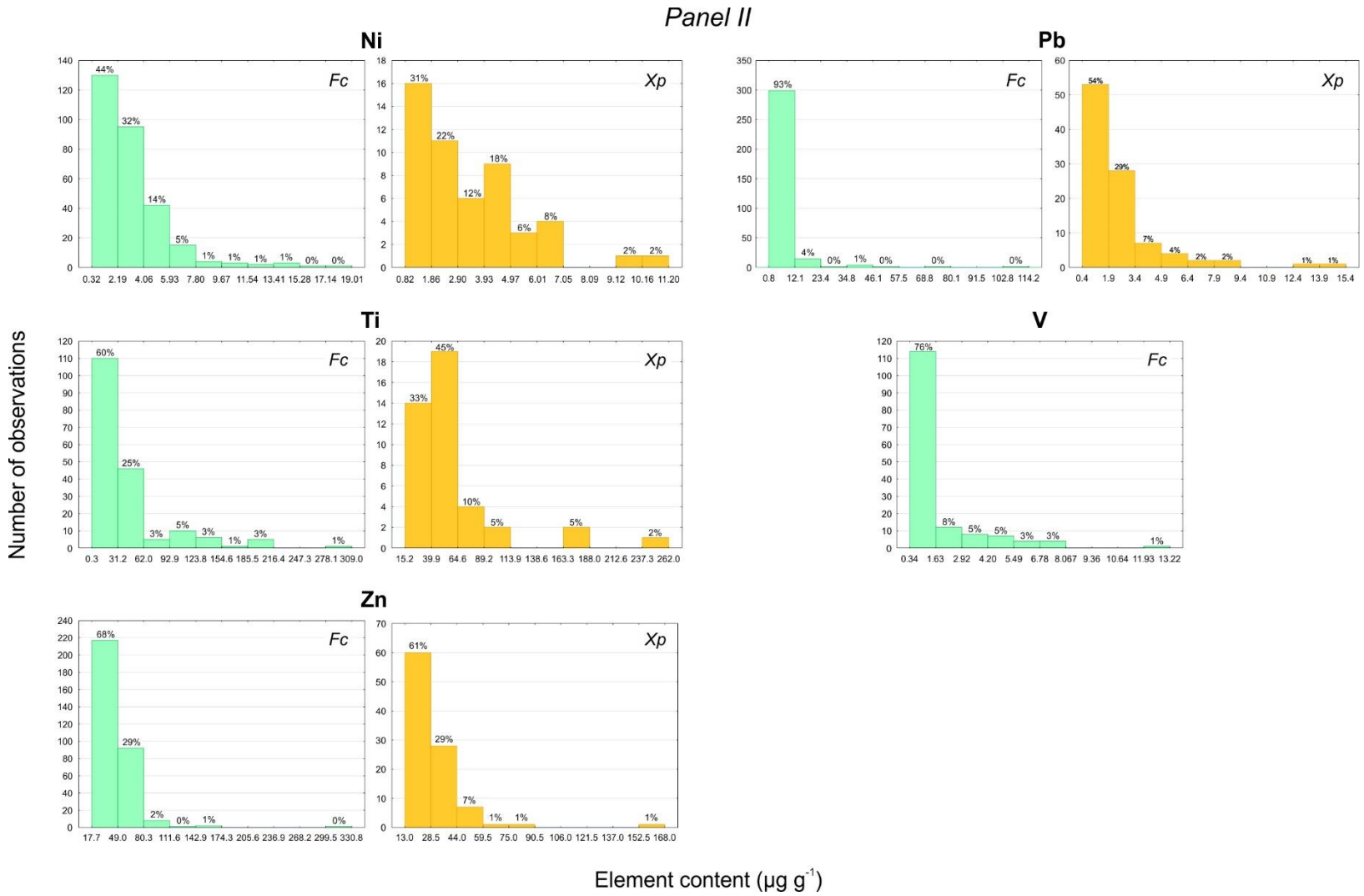
Supplementary Table S6. Results of the comparative application of the accumulation/loss scale by Frati et al. (2005) (Acc./loss classes) and the bioaccumulation scale in Table 5 (B. class) to a real case study. Data refer to three elements (As, Cd, Cr) measured in samples of *Pseudevernia furfuracea* collected in 30 transplant sites (Fig. 4). Abbreviations for naturality/alteration and bioaccumulation classes are reported in Supplementary Table S4 and Table 5. The attribution of classes is based on the *EU* ratio (former *EC* ratio) for both scales.

Site	As				Cd				Cr			
	Element concentration	*EU* ratio	Acc./loss class	B. class	Element concentration	*EU* ratio	Acc./loss class	B. class	Element concentration	*EU* ratio	Acc./loss class	B. class
A1	0.26	1.06	N.	2 (L)	0.23	1.21	N.	2 (L)	1.74	1.35	Acc.	2 (L)
A2	0.26	1.02	N.	2 (L)	0.46	2.44	S.a.	3 (M)	1.59	1.23	N.	2 (L)
A3	0.32	1.26	Acc.	2 (L)	0.23	1.22	N.	2 (L)	2.35	1.82	S.a.	3 (M)
A4	0.25	0.98	N.	2 (L)	0.21	1.10	N.	2 (L)	1.53	1.19	N.	2 (L)
A5	0.37	1.47	Acc.	2 (L)	0.46	2.44	S.a.	3 (M)	2.38	1.84	S.a.	3 (M)
A6	0.27	1.10	N.	2 (L)	0.25	1.34	Acc.	2 (L)	1.85	1.44	Acc.	2 (L)
B1	0.31	1.24	N.	2 (L)	0.25	1.30	Acc.	2 (L)	1.46	1.13	N.	2 (L)
B2	2.34	9.35	S.a.	5 (S)	0.17	0.90	N.	1 (A)	1.46	1.14	N.	2 (L)
B3	0.28	1.12	N.	2 (L)	0.22	1.14	N.	2 (L)	1.85	1.43	Acc.	2 (L)
B4	0.32	1.28	Acc.	2 (L)	0.26	1.35	Acc.	2 (L)	1.35	1.05	N.	2 (L)
B5	0.29	1.16	N.	2 (L)	0.30	1.60	Acc.	2 (L)	2.02	1.57	Acc.	2 (L)
B6	0.27	1.07	N.	2 (L)	0.23	1.21	N.	2 (L)	1.40	1.09	N.	2 (L)
B7	0.33	1.31	Acc.	2 (L)	0.28	1.48	Acc.	2 (L)	1.63	1.26	Acc.	2 (L)
B8	0.33	1.31	Acc.	2 (L)	0.24	1.29	Acc.	2 (L)	2.09	1.62	Acc.	2 (L)
C1	0.37	1.48	Acc.	2 (L)	0.28	1.49	Acc.	2 (L)	2.37	1.83	S.a.	3 (M)
C2	0.35	1.38	Acc.	2 (L)	0.24	1.29	Acc.	2 (L)	2.09	1.62	Acc.	2 (L)
C3	0.22	0.88	N.	1 (A)	0.21	1.13	N.	2 (L)	1.16	0.90	N.	2 (L)
C5	0.31	1.26	Acc.	2 (L)	0.30	1.60	Acc.	2 (L)	1.60	1.24	N.	2 (L)
C6	0.29	1.15	N.	2 (L)	0.20	1.07	N.	2 (L)	1.81	1.41	Acc.	2 (L)
D1	0.21	0.84	N.	1 (A)	0.20	1.03	N.	2 (L)	1.65	1.28	Acc.	2 (L)
D2	0.22	0.90	N.	1 (A)	0.21	1.10	N.	2 (L)	1.33	1.03	N.	2 (L)
D3	0.29	1.15	N.	2 (L)	0.44	2.31	S.a.	3 (M)	2.03	1.57	Acc.	2 (L)
D4	0.23	0.93	N.	2 (L)	0.21	1.11	N.	2 (L)	1.19	0.92	N.	2 (L)
D5	0.37	1.50	Acc.	2 (L)	0.40	2.09	S.a.	3 (M)	2.06	1.60	Acc.	2 (L)
D6	0.24	0.97	N.	2 (L)	0.25	1.33	Acc.	2 (L)	1.69	1.31	Acc.	2 (L)
E1	0.24	0.95	N.	2 (L)	0.27	1.41	Acc.	2 (L)	1.75	1.36	Acc.	2 (L)
E2	0.25	1.02	N.	2 (L)	0.22	1.15	N.	2 (L)	1.63	1.26	Acc.	2 (L)
E3	0.25	1.01	N.	2 (L)	0.27	1.41	Acc.	2 (L)	1.54	1.20	N.	2 (L)
E4	0.22	0.90	N.	1 (A)	0.22	1.14	N.	2 (L)	1.76	1.37	Acc.	2 (L)
E5	0.22	0.90	N.	1 (A)	0.19	0.99	N.	2 (L)	1.28	0.99	N.	2 (L)

SUPPLEMENTARY FIGURES S1-S3



Supplementary Figure S1. Distributions of element concentration data for *Flavoparmelia caperata* (*Fc*: pale green bars) and *Xanthoria parietina* (*Xp*: orange bars) in the dataset *N* (panel I: Al, As, Cd, Cr, Cu, Hg; panel II: Ni, Pb, Ti, V, Zn).



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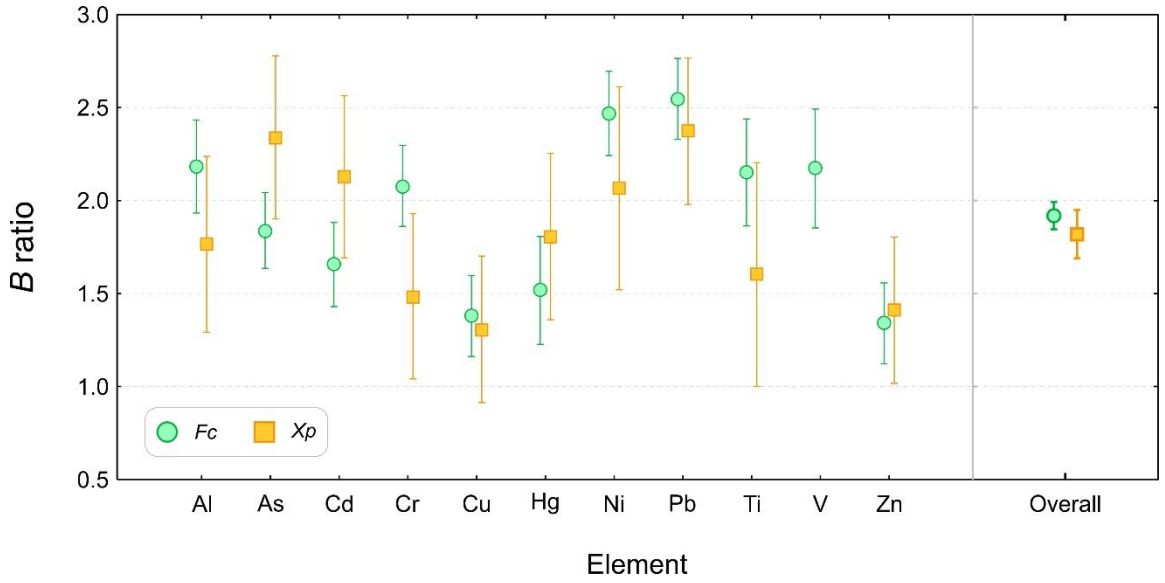
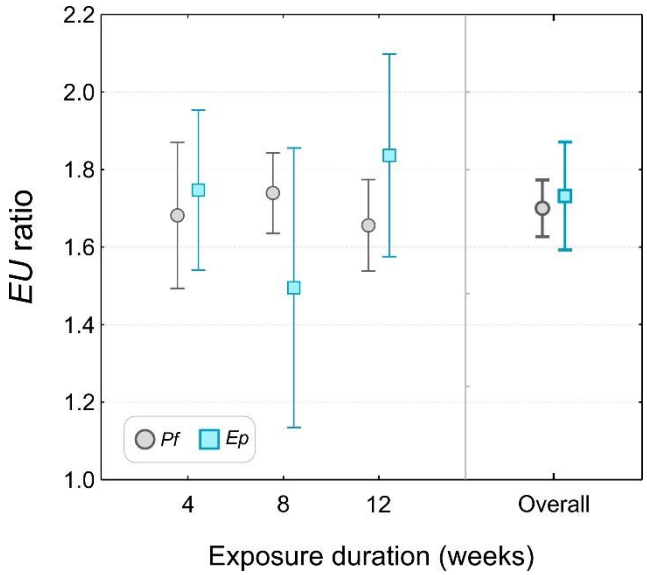


Supplementary Figure S2. *B* ratio data, separately reported for 11 elements or not (overall), in the lichen species *Flavoparmelia caperata* (*Fc*: pale green) and *Xanthoria parietina* (*Xp*: orange). Data are shown as means and 95% confidence intervals.



Supplementary Figure S3. *EU* ratio data, separately reported for three exposure time spans (4, 8 and 12 weeks) or not (overall), in the lichen species *Evernia prunastri* (*Ep*: pale blue) and *Pseudevernia furfuracea* (*Pf*: grey). Data are shown as means and 95% confidence intervals.

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PART 2

METHODOLOGICAL ISSUES IN PRACTICAL APPLICATIONS

Beyond ozone-tolerance: Effects of ozone fumigation on trace element and PAH enriched thalli of the lichen biomonitor *Pseudevernia furfuracea*

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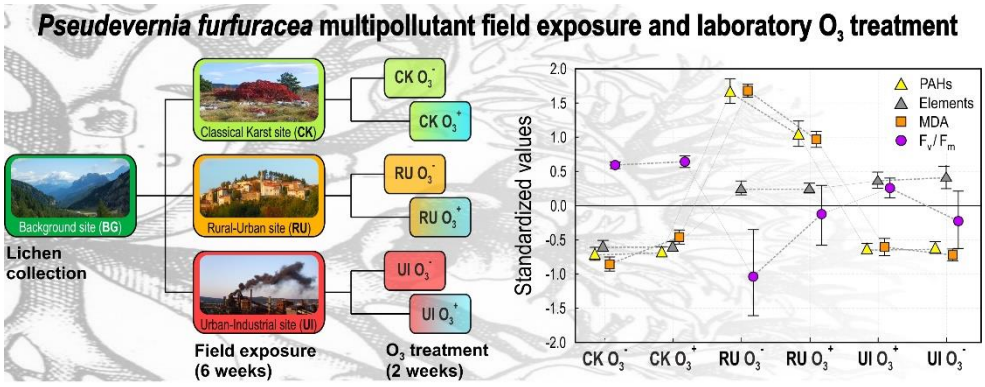
Highlights

• The response of the lichen *Pseudevernia furfuracea* to O_3 was investigated.
• *P. furfuracea* was subjected to multi-pollutant field exposure and O_3 treatment.
• The physiological impairment of the chlorolichen due to O_3 was rather limited.
• O_3-driven depletion of PAHs accumulated by thalli in the field cannot be excluded.

Abstract

In this study, the effects of ozone (O_3) on the physiology of the lichen *Pseudevernia furfuracea* var. *furfuracea* previously subjected to field stressing conditions were assessed. Samples collected in a pristine site were exposed for 6 weeks at 3 sites characterized by different pollution, *e.g.* elemental and PAH depositions (site RU, close to wood-burning house chimneys; site UI, close to cast-ironworks; site CK, in a semi-natural context). Afterwards, samples were transferred to controlled fumigation chambers, where they were either O_3-treated for 2 weeks (250 ppb O_3 for 5 h day^{-1}, O_3^+ samples) or not (0 ppb O_3, O_3^- samples). Three physiological markers (F_v/F_m, maximum quantum yield of primary photochemistry; MDA, malondialdehyde content; potassium leakage) as well as elemental and PAH concentrations were measured in matched sets of sample replicates at each experimental step. Data were explored by multivariate techniques and the effects of field exposure and fumigation were tested by generalized linear models (GLM). Detrimental effects on MDA and F_v/F_m were observed limited to samples exposed in RU and UI sites. Physiological parameters in O_3-treated samples showed heterogeneous variation patterns with respect to field-exposed ones. A recovery of F_v/F_m was observed in RU- and UI-exposed samples, whereas a significant increase of MDA was highlighted limited to CK O_3^+ and CK O_3^- samples, possibly related to a "chamber effect". Overall, the impairment caused by ozonation was limited, proving the strong O_3-tolerance of our test species. Interestingly, the content of the most abundant 4-ring PAHs in RU O_3^+ samples, which underwent the highest field enrichment of PAHs, was significantly lower than that of matched RU O_3^- samples. This suggested a possible role of ozone in degrading PAHs at thallus level, with interesting interpretative repercussions in the context of transplant-based surveys aimed at evaluating PAH depositions when O_3 ground levels are high.



Keywords: air pollution; O_3; bioaccumulation; malondialdehyde; chlorophyll *a* fluorescence; potassium leakage.

Abbreviations:

Ace: acenaphthene;

Acy: acenaphthylene;

Ant: anthracene;

B[a]Ant: benzo[a]anthracene;

B[ah]Ant: dibenzo[a:h]anthracene;

B[a]Py: benzo[a]pyrene;

B[b]Fl: benzo[b]fluoranthene;

B[e]Py: benzo[e]pyrene;

B[ghi]Per: benzo[g:h:i]perylene;

B[j+k]Fl: benzo[j+k]fluoranthene;

Chry: chrysene;

F: fluorene;

Fl: fluoranthene;

I[cd]Py: indeno[1:2:3-cd]pyrene;

N: naphthalene;

P: phenanthrene;

Py: pyrene.

1. Introduction

Tropospheric ozone (O_3) is a strongly oxidizing secondary air pollutant and greenhouse gas (Logan 1985; Wu et al. 2008), formed by a series of photochemical reactions between nitrogen oxides (NO_x) and volatile organic compounds (Hassan et al. 2013; Paoletti et al. 2017; Lefohn et al. 2018), and favoured by high temperatures. Ozone annual averages are increasing at both urban and rural sites, being an important component of global change (Paoletti et al. 2014).

In heavily polluted areas, ozone exposure effects are acknowledged at physiological, biochemical and molecular level (Goumenaki et al. 2010) on several organisms, including humans (Chen et al. 2017; Nuvolone et al. 2018) and plants (Nali et al. 2007; Paoletti 2007; Saitanis 2008; Sarkar et al. 2010). The O_3-sensitivity of plants is highly variable (Schraudner et al. 1998) and mostly related to their ability in detoxifying Reactive Oxygen Species (ROS; Frei et al. 2010). Several ROS detoxification mechanisms are known to occur in plants, and some of these are even acknowledged as variety-specific. A case in point in this regard is *Nicotiana tabacum*, with its O_3-supersensitive and O_3-tolerant cultivars (Bel-W3 and Bel-B, respectively), that allowed the development of a standardized protocol to biomonitor ambient air O_3 concentrations (EN 16789:2016) as well as to produce miniaturized plantlet kits for outdoor O_3 biomonitoring (Lorenzini 1994; Nali et al. 2007; Lorenzini and Nali 2018).

Besides plants, the effects of O_3 were also investigated in lichens. These symbiotic organisms, widely used as biomonitors of atmospheric pollution, are sensitive to a variety of gaseous pollutants, but their response to O_3 is still debated. Field studies carried out along oxidant gradients, coupled with the analysis of herbarium samples, highlighted residual amounts or no occurrence of once abundant species (mostly cyanolichens: *Collema nigrescens*, *Peltigera* spp., *Pseudocyphellaria* spp., but also chlorolichens of the genus *Usnea*) at sites that experienced a substantial increase of O_3 concentrations (Sigal and Nash 1983; Nash 2008). However, several recent studies carried out in open top chambers and fumigation chambers highlighted a noticeable O_3-tolerance of chlorolichens for a wide range of O_3 concentrations [(0-)10-250(-50˙000) ppb]. In these studies, the lichen response to O_3 was generally investigated (*i*) in fully or partially controlled environments (*e.g.*, Brown and Smirnoff 1978; Nash and Sigal 1979; Ross and Nash 1983; Sigal and Johnston 1986; Scheidegger and Schroeter 1995; Tarhanen et al. 1997; Riddell et al. 2010; Bertuzzi et al. 2013, 2018; Pellegrini et al. 2014; Vannini et al. 2018), with the rationale of controlling/minimizing the effects related to accessory environmental causes of physiological stress, and (*ii*) directly in the field, "*en plein air*", with the aim of assessing and/or disentangling the physiological effects caused by O_3 when co-occurring with other gaseous pollutants (such as NO_x, gaseous HNO_3 and SO_2; Egger et al. 1993; Riddell et al. 2012; Tretiach et al. 2012). The evaluation of composite effects of pollutants on the physiology of biomonitors is receiving increasing interest in the last years, because there is an urgent need to study the behaviour of biomonitors in response not to single xenobiotics, but to their naturally occurring mixtures (*e.g.*, Sujetovienė and Galinytė 2016). This knowledge is in fact necessary to correctly convert the information they can give into air quality assessment.

The fruticose macrolichen *Pseudevernia furfuracea* var. *furfuracea* (L.) Zopf. is a bioaccumulator of extensive use in active biomonitoring (*e.g.*, Sloof 1995; Tretiach et al. 2011;

Nascimbene et al. 2014; Kodnik et al. 2015), being locally abundant (Cecconi et al. 2018) and tolerant to several gaseous phytotoxic pollutants (Miszalski and Niewiadomska 1993; Tretiach et al. 2007; Malaspina et al. 2018), on account of an efficient antioxidant machinery, that permits the survival of this species in environments characterized by high UV, high light, and low temperatures. In a study based on field fumigation chambers, this chlorolichen was defined as O_3-tolerant by Scheidegger and Schroeter (1995), because no detrimental effects were observed either at ultrastructural or at functional level (chlorophyll a fluorescence, Chl_aF, being used as a proxy of photosystem functionality). In their experiments, as done in the majority of the above cited works, the authors used healthy samples purposely collected in "pristine" environments.

Aim of this study was to evaluate the response to O_3 of *P. furfuracea* thalli, still collected in "pristine" environments as done by Scheidegger and Schroeter (1995), but preliminary subjected to a mixture of multi-origin pollutants and thus enriched in (*e.g.*) heavy metal-rich particulate matter, PAHs, etc. Our test hypothesis was that previously field-stressed thalli would exhibit physiological impairment due to subsequent ozonation treatment. The physiological status of samples was assessed after each experimental step using a multi-marker approach encompassing both symbiotic partners. Biomarkers included the chlorophyll *a* fluorescence (Chl_aF), the content of malondialdehyde (MDA), and the leakage of potassium ions (K^+ leakage), respectively considered as proxies of photosynthetic activity of algal population, peroxidation of membrane lipids, and membrane integrity.

2. Materials and methods

2.1 Lichen material, collection and sample pre-processing

Pseudevernia furfuracea is a meso-xerophilous lichen, growing on nutrient-poor, acid bark substrata (Nimis 2016). The species is spread in cool temperate areas, where it may be locally very common (Rikkinen 1997; Smith et al. 2009). The dorsi-ventral thallus is richly branched with upper surfaces often covered by finger-shaped outgrowths (isidia), which considerably increase the exchange surface per area and mass unit (Tretiach et al. 2005) and the particle entrapment (Bargagli and Mikhailova 2002). The large thallus size and the easy identification in the field ensure fast sampling and preparation. *P. furfuracea* has also been targeted in several methodological studies, aimed at providing standardized methodologies (*e.g.*, Adamo et al. 2007, 2008; Incerti et al. 2017) and improving data quality in biomonitoring (Cecconi et al. 2018).

Thalli of *P. furfuracea* var. *furfuracea* were collected from isolated larch trees (*Larix decidua* L.) in a background area (henceforth BG; Cecconi et al. 2018) of the Carnic Alps (Lateis, NE Italy) at 1500 m a.s.l. Thalli, still attached to *c.*15-20 cm long twigs, were transported to the laboratory in paper bags and left to dry out in dim light at room temperature for 24 h. The lichen material was carefully cleaned from bark fragments, debris and other lichen and moss species. Moderately isidiate thalli of comparable size and branching, without sexual reproductive structures, were selected for the experimentation (Tretiach et al. 2007, 2011; Incerti et al. 2017). Different sets of 5 sample replicates each were obtained from the bulk lichen material with the aim of assessing physiological parameters, elemental and PAH content before the experimental treatments (henceforth 'pre-exposure' or BG samples): these samples were dehydrated in silica gel for 48 h,

vacuum sealed and stored at -20 °C until the end of the experiment. The remaining lichen material was mounted on exposure devices. A single exposure device consisted in a 120 cm long wooden rod bearing thalli still attached on their twigs in a sufficient amount to build up sets of 3 sample replicates to assess physiological parameters, elemental and PAH content after field exposure and subsequent fumigation.

2.2 Experimental design: field exposure and O_3 fumigation

In order to evaluate the physiological response of our test species to O_3, a double-step experiment was planned. Firstly, *P. furfuracea* thalli were exposed for 6 weeks at three sites with different pollutant loads (mostly elemental and PAH depositions). The field exposure was followed by 2 week-stay in fumigation chambers, where samples were either O_3-treated or not. At each experimental step (*i.e.*, before and after the field exposure and after the controlled fumigation), the set of selected physiological markers, as well as elemental and PAH concentrations were measured in matched sets of sample replicates.

The field exposure was carried out in the Trieste province (NE Italy), between February 18th and April 4th, 2016 in order to avoid summer-like ambient O_3 levels (Nali et al. 2007). Lichen samples, mounted on the exposure devices, were transplanted at three sites, characterised by different land use: i) a semi-natural site in the Classical Karst (CK), far from major sources of anthropogenic pollution; ii) a rural-urban site (RU) in the Classical Karst, where the lichen samples were purposely placed close to wood-burning house chimneys with intense activity; iii) an urban-industrial site (UI) in the city of Trieste, close to a large operating cast-ironwork (ARPA FVG 2018a; Supplementary Fig. S1). During field exposure, air temperature and relative humidity were continuously collected at the three sites by EL-USB-2 data loggers. Exposure devices were secured to artificial supports at 4 m above the ground. After 6 weeks, lichen samples were retrieved, sealed, and transported in cool bags to the laboratory. A first set of 3 samples per exposure site was immediately processed to measure the physiological parameters (sect 2.4), while a second set was processed and stored for the determination of elemental and PAH content (Sect. 2.3) at the end of the experiment (field-exposed samples; Table 1). Further two matched paired groups of 3 samples per exposure site were sealed and transported to University of Pisa, where the fumigation was carried out.

Table 1. List of experimental factors and description of their levels.

Experimental factor		Levels and description
Field exposure (*Exp.*)	CK	Samples exposed in the proximate-natural site in the Classical Karst.
	RU	Samples exposed in the rural-urban site, close to wood-burning house chimneys.
	UI	Samples exposed in the urban-industrial sites, close to an operating cast-ironworks.
Fumigation (*Fum.*)	CK O_3^+ RU O_3^+ UI O_3^+	Samples exposed in the field sites, then ozonated in fumigation chambers.
	CK O_3^- RU O_3^- UI O_3^-	Samples exposed in the field sites, non-ozonated but kept in fumigation chambers at the same environmental conditions of O_3^+ samples.

Samples were placed for 2 weeks in a ventilated 0.90 × 0.90 × 0.65 cm Perspex chamber with the inlet air (two complete air changes min^{-1}) either subjected or not to 250 ± 4 ppb O_3 (herein, the

notation O_3^+ and O_3^- refers to samples either O_3-treated or not; Table 1), provided for 5 h day^{-1} in form of a square wave generated by a Fisher 500 air-cooled apparatus (Zurich, CH) supplied with pure oxygen. The sample watering, necessary to secure a minimum metabolism for *c*. 3 h day^{-1}, was performed by spraying *c*. 0.01 mL cm^2 dH_2O in the morning, immediately before the input of O_3 (Bertuzzi et al. 2018). A photosynthetic photon flux density (PPFD) of 60 µmol photon m^{-2} s^{-1} was provided for 12 hours day^{-1} by four quartz metal halide lamps with clear outer bulb (400 W, MASTER HPI-T Plus, Philips, NL) and by four high-pressure sodium lamps with clear tubular outer bulb (250 W, SON-T, Philips, NL).

After fumigation, samples were dried at room temperature in dim light for 12 h. Consequently, O_3^+ and O_3^- samples were processed to assess the physiological parameters, elemental and PAH content as described below.

2.3 Analytical procedures

Pre-exposure (BG), field-exposed (CK, RU, UI) and fumigated (CK O_3^-, CK O_3^+, RU O_3^-, RU O_3^+, UI O_3^-, UI O_3^+) samples were singly processed for the determination of elemental and PAH content.

2.3.1 Elemental content

Samples earmarked for elemental analysis were dried out for 12 h, and terminal lobes of 2.5 cm were selected to assemble 1 g samples, which were pulverized with a planetary ball mill (Retsch PM100); the resulting powder was dried overnight at 40 °C and stored in microtubes (Bargagli and Nimis 2002). Afterwards, the batch was submitted to the determination of the content of 25 elements (Al, As, Ba, Ca, Cd, Cr, Cu, Fe, K, Li, Mg, Mo, Mn, Na, Ni, P, Pb, S, Sb, Sc, Se, Sn, Ti, V, Zn). Replicate splits of 0.25 g were digested in a HNO_3-$HClO_4$-HF solution until fuming and then dried; the resulting residue was dissolved in 50% HCl solution and heated. The elemental content was determined by a Perkin Elmer Elan 6000 ICP mass spectrometer and all the values were expressed on a dry weight (DW) basis (µg g^{-1}). Accuracy was expressed in terms of mean recovery percentages, calculated as the ratio between the certified concentration values for the standard reference material BCR 482 (Quevauviller et al. 1996) and those measured in aliquots of the same standard blindly included in the batch (Supplementary Table S1).

2.3.2 PAHs

Samples earmarked for PAH analysis were dried out for 12 h, and terminal lobes of 2.5 cm were selected to assemble 1.5 g samples, which were finely chopped with ceramic scissors, sealed in glass jars and kept in the dark at 4 °C until analytical determination (Augusto et al. 2013). The content of 16 EPA priority PAHs (*i.e.*, Ace, Acy, Ant, F, P, Chry, Fl, Py, B[a]Ant, B[ah]Ant, B[a]Py, B[b]Fl, B[e]Py, B[j+k]Fl, I[cd]Py, B[ghi]Per) plus B[e]Py was measured. Prior to analysis, PAHs were extracted from lichen samples using 30 mL of a mixture of hexane/dichloromethane (1:1) in a Milestone Start E Microwave Extraction System, according to the US EPA method 3546 (EPA 2018a). Subsequently, samples were purified by Supelclean™ LC-NH_2 SPE tube (bed wt. 500 mg), filtered, concentrated to 1 mL and further evaporated. The PAH content was determined by means of gas chromatography-mass spectrometer (GC-MS triple quadrupole, Bruker, model

TQ300), in accordance to the US EPA method 8270 (EPA 2018b). All the values were expressed on DW basis (ng g^{-1}). Accuracy of the analytical procedure was assessed in terms of measured-to-expected concentrations of deuterated PAH standards added to the experimental samples prior to the extraction.

2.4 Physiological measurements

The physiological status of lichen samples was assessed by measuring the chlorophyll *a* fluorescence (Chl$_a$F), the content of malondialdehyde (MDA), and the leakage of potassium ions (K^+ leakage).

2.4.1 Chlorophyll a *fluorescence*

Chl$_a$F was assessed in terms of the maximum quantum yield of primary photochemistry in dark adapted samples (F_v/F_m) and non-photochemical quenching (NPQ). Measurements were carried out on 6 lobes (60 ± 5 mg each) per sample, randomly selected for each experimental group. Before Chl$_a$F measurements, samples were hydrated in a glass jar at 100% relative humidity (RH) for 24 h (PPFD, 29 ± 2 µmol photons m^{-2} s^{-1}; 18 ± 1 °C; 12 h dark / 12 h light) and consequently rinsed for 3 minutes in dH_2O. Afterwards, selected lobes were gently shaken to remove the excess of dH_2O and dark-adapted for 30 minutes. Chl$_a$F measurements were taken with a pulse-amplitude-modulated fluorometer PAM-2000 (Walz, Effeltrich), positioning the measuring fibre at 60° on the upper surface of the lobes. The modulated light was turned on to obtain the minimal Chl$_a$F level (F_0). A saturating light pulse of *c*. 8000 µmol photons m^{-2} s^{-1} for 0.8 s was emitted to obtain the transient maximum Chl$_a$F level (F_m) and thus to calculate the variable Chl$_a$F level (F_v, *i.e.* F_m - F_0) and the maximum quantum efficiency of PSII photochemistry (F_v/F_m). An external actinic light provided by a light unit FL-460 (Walz, Effeltrich, D) with a halogen lamp was turned on to record the Kautsky effect at an intensity of 176 µmol photons m^{-2} s^{-1}. Such value is consistent with the species-specific $PPFD_{lk}$ value, *i.e.* the photosynthetic photon flux at which the quantum yield of CO_2 assimilation is the highest (Piccotto and Tretiach 2010). Once the emission peak was achieved (F'_m), saturating light pulses were applied at 60 s intervals during actinic illumination to determine NPQ (see *e.g.*, Baker 2008; Bussotti et al. 2011). NPQ was calculated as (F_m - F'_m) / F'_m. Chl$_a$F measurements were repeated on the same lobes after 48 h recovery, at 100% RH and 29 µmol photons m^{-2} s^{-1}.

2.4.2 MDA assay

Lipid peroxidation was determined by the thiobarbituric acid reactive substances (TBARS) assay following the method proposed by Candotto Carniel et al. (2017) and based on Heath and Packer (1968). Three lobe samples (200 mg each) for each experimental group were pulverized with liquid nitrogen, lyophilized, homogenized in a mortar using 1.5 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 12000*g* for 20 minutes at room temperature. An aliquot of 0.5 mL of the supernatant was collected and mixed with 1 mL of 20% TCA with 0.5% thiobarbituric acid (TBA). The mixture was heated at 95 °C for 25 minutes, quickly cooled in an ice bath and centrifuged at 15000*g* for 10 minutes at room temperature. The supernatant was removed and used to determine MDA concentration. Absorbance readings were taken at 532 nm using a Jenway 7315 UV-vis

spectrophotometer (Stone, UK) and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The amount of MDA was calculated by using a molar extinction coefficient of 155 mM^{-1} cm^{-1} and the results were expressed as nmol g^{-1} (DW).

2.4.3 K^+ leakage

In order to investigate membrane damage, K^+ leakage was measured on 3 lobes (60 ± 5 mg each) per sample, randomly selected for each experimental group. K^+ leakage was measured following the method proposed by Candotto Carniel et al. (2017). Terminal lobes were rinsed in 25 mL of dH_2O and continuously agitated at 100 rpm for 60 minutes. Afterwards, lobes were removed from suspensions, oven-dried at 80 °C for 24 h and weighed. The remaining dH_2O was filtered (0.45 µm) and stored at 4 °C until analysis. Acqueous K^+ concentration was determined by flame atomic adsorption spectroscopy (Perkin Elmer Analyst 400) with an uncertainaty less than 2%. The limit of detection (LOD) at the operative wavelength of 766.5 nm was 0.01 mg l^{-1}. Microtubes with dH_2O only were used as blanks. K^+ leakage was expressed as mg of leached K^+ per g (DW) of lichen lobes.

2.5 Data analysis

Data were organized in a matrix of 27 cases (3 replicates per 9 experimental groups) × 34 variables. Variables included 25 chemical elements (Sect. 2.3.1), 5 PAH categories (2-, 3-, 4-, 5- and 6-rings), the sum of all PAHs (Σ PAHs), and 3 physiological parameters (F_v/F_m, MDA and K^+ leakage). Amongst the fluorescence parameters, NPQ was not included due to its high correlation with F_v/F_m values (Spearman's rho 0.91, $p < 0.01$). The matrix was preliminarily submitted to explorative multivariate analysis. In particular, a hierarchical Cluster Analysis (CA) was performed on the data matrix after a classic standardization of values (standardized values were obtained from the original values by subtraction of the mean of all samples, and dividing the result by the standard deviation), in order to ensure the full commensurability of variables (Podani 2007). CA was performed for the set of 25 elements with Euclidean distance as distance measure and Ward's method as grouping algorithm. A Principal Component Analysis (PCA) based on correlations among the variables was performed on the data matrix reporting non-standardized values. In the PCA, the land use classes of the exposure sites (Table 1) were plotted as supplementary variables following the approach suggested by Legendre and Legendre (1998). In addition, significant differences among physiological parameters in experimental samples were tested using Kruskal-Wallis ANOVA and non-parametric Dunn's post hoc test (Dinno 2017).

In order to disentangle the effects of the field exposure and O_3-treatment on the physiological state of lichen thalli, generalized linear models (GLMs) were fitted, limited to the data matrix of paired fumigated sample replicates. Main and interactive effects of field exposure (fixed effect with 3 levels, Table 1) and fumigation (fixed effect with 2 levels, Table 1) were tested, considering physiological variables, PAH and elemental content as dependent variables. In particular, standardized values for each target variable were used, separately considering each value as an individual observation.

All data analyses and graphics were performed with the software packages Statistica v. 10 (StatSoft Inc., Tulsa, OK, USA) and R (R Core Team, 2013). Statistical significance was tested at $\alpha = 0.05$ in all cases.

3. Results

3.1 Trace element and PAH enrichment in field-exposed thalli

Samples exposed for 6 weeks in the field sites CK, RU and UI (Supplementary Fig. S1) exhibited different PAH and elemental content.

Expectedly, samples exposed in the semi-natural site CK did not show significant variation with respect to background situation for the overall PAH and element content as well as for each of the three element clusters, as identified by the CA (Supplementary Fig. S2). On the contrary, samples exposed in site RU showed an increase in elemental content, especially for the elements of cluster I (Ca, Cd, Mg, Na, Sc and V; Fig. 1), but also a noteworthy increase of PAHs (except for PAH-2; see also post hoc results for PAH content in Table 2). Samples exposed in site UI also exhibited increased PAH and elemental content, however the PAH accumulation was less pronounced than in RU samples; in site UI, the highest elemental enrichment was observed for elements of cluster II (Al, Ba, Cr, Fe, Li, Mo, Pb, S, Se, Ti and Zn; Fig. 1). Overall, the elemental enrichment in both RU and UI samples was rather limited. Indeed, when expressing the element concentration data of Exposed samples (*E*) with respect to that of Unexposed samples (*U*) in terms of their ratio (*i.e.*, the so-called *EU* ratio, Cecconi et al. 2019), only few elements exceeded the value of 1.5 (*i.e.*, elemental content increased more than 50% in exposed samples). Namely, Na highly accumulated in all the exposure sites (2.72, 7.60 and 4.07 in CK, RU and UI samples, respectively), Ti and Sb (1.56 and 1.59) in site RU, and Al, Fe and Sb (1.67, 3.67 and 1.83) in site UI.

Table 2. Mean and standard deviation of the target variables (physiological parameters and PAH content) in different groups of samples (labelled as in Table 1) of the epiphytic lichen *Pseudevernia furfuracea* var. *furfuracea* (n = 5 for BG group, n = 3 for the rest of groups). Target variables include malondialdehyde content (MDA; nmol g^{-1}), potassium leakage (K^+ leakage; mg g^{-1}), maximum quantum efficiency of photosystem II (F_v/F_m), the sum of 18 polycyclic aromatic hydrocarbons as well as the sum of PAHs with 2-, 3-, 4-, 5- and 6-rings (respectively, Σ PAHs, PAH-2, PAH-3, PAH-4, PAH-5 and PAH-6; expressed in ng g^{-1}). Following one-way ANOVA, different letters indicate significantly different groups within each column (Dunn's post hoc test at $p < 0.05$).

Group	Physiological markers			PAH content					
	MDA	K^+ leakage	F_v/F_m	Σ PAHs	PAH-2	PAH-3	PAH-4	PAH-5	PAH-6
BG	15.9 ± 0.9 ^{a}	0.131 ± 0.023 ^{ab}	0.719 ± 0.009 ^{c}	200.2 ± 129.1 ^{a}	22.8 ± 30.0 ^{a}	79.3 ± 54.8 ^{a}	68.8 ± 57.3 ^{a}	22.0 ± 12.4 ^{ab}	7.3 ± 3.9 ^{a}
CK	12.5 ± 1.2 ^{a}	0.124 ± 0.014 ^{ab}	0.700 ± 0.021 ^{c}	245.5 ± 76.1 ^{a}	39.9 ± 13.3 ^{a}	96.8 ± 21.0 ^{a}	93.2 ± 39.8 ^{ab}	11.9 ± 4.3 ^{a}	3.8 ± 0.1 ^{a}
RU	56.4 ± 5.9 ^{d}	0.224 ± 0.013 ^{b}	0.505 ± 0.090 ^{a}	4023.2 ± 1164.4 ^{c}	30.1 ± 10.8 ^{a}	1374.5 ± 341.0 ^{c}	2510.5 ± 800.3 ^{d}	92.8 ± 24.5 ^{c}	15.3 ± 7.4 ^{b}
UI	24.5 ± 1.9 ^{bc}	0.145 ± 0.094 ^{ab}	0.593 ± 0.034 ^{ab}	813.4 ± 191 ^{b}	88.3 ± 22.5 ^{a}	309.2 ± 39.6 ^{b}	368.9 ± 128.0 ^{ab}	38.8 ± 24.9 ^{b}	8.2 ± 5.8 ^{ab}
CK O_3^-	21.6 ± 1.1 ^{b}	0.105 ± 0.016 ^{ab}	0.708 ± 0.007 ^{c}	185.4 ± 23.7 ^{a}	15.9 ± 7.2 ^{a}	82.9 ± 4.3 ^{a}	58.7 ± 4.2 ^{a}	21.6 ± 11.5 ^{ab}	7.1 ± 5.7 ^{a}
CK O_3^+	29.3 ± 0.6 ^{c}	0.093 ± 0.073 ^{a}	0.713 ± 0.013 ^{c}	206.0 ± 20.8 ^{a}	13. 7 ± 7.5 ^{a}	75.1 ± 17.1 ^{a}	89.2 ± 12.9 ^{ab}	22.7 ± 5.9 ^{ab}	6.2 ± 4.2 ^{a}
RU O_3^-	71.2 ± 2.3 ^{e}	0.161 ± 0.059 ^{ab}	0.569 ± 0.178 ^{ab}	1492.7 ± 169.5 ^{b}	31.9 ± 1.8 ^{a}	567.1 ± 85.1 ^{b}	856.1 ± 61.7 ^{c}	39.0 ± 24.6 ^{ab}	6.9 ± 5.3 ^{a}
RU O_3^+	57.3 ± 3.9 ^{d}	0.199 ± 0.137 ^{ab}	0.646 ± 0.065 ^{bc}	1152.7 ± 175.6 ^{b}	35.1 ± 29.4 ^{a}	558.8 ± 41. 5 ^{b}	530.2 ± 122.4 ^{b}	22.6 ± 11.4 ^{ab}	7.0 ± 5.5 ^{a}
UI O_3^-	26.4 ± 4.3 ^{bc}	0.205 ± 0.061 ^{ab}	0.680 ± 0.021 ^{bc}	234.5 ± 22.1 ^{a}	21.6 ± 6.6 ^{a}	86.9 ± 7.8 ^{a}	105.6 ± 15.3 ^{ab}	17.6 ± 7.8 ^{ab}	3.8 ± 0.1 ^{a}
UI O_3^+	24.0 ± 0.8 ^{b}	0.123 ± 0.079 ^{ab}	0.640 ± 0.062 ^{bc}	236.1 ± 17.3 ^{a}	21.3 ± 7.0 ^{a}	90.5 ± 13.2 ^{a}	110.9 ± 18.7 ^{ab}	10.5 ± 1.8 ^{a}	3.6 ± 0.2 ^{a}

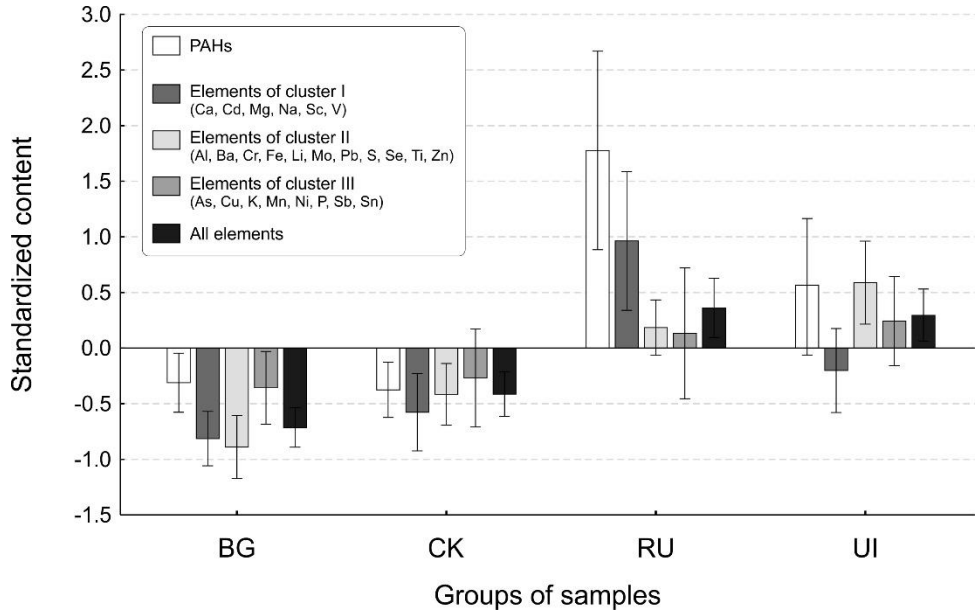


Figure 1. Standardized PAH and elemental content (separately reported for the 3 element clusters derived from CA, and for all the 25 elements) in samples of the epiphytic lichen *Pseudevernia furfuracea* var. *furfuracea* collected in a background site (BG) and transplanted at the exposure sites (CK, Classical Karst; RU, rural-urban site; UI, urban-industrial site). Data are shown as mean and 95% confidence interval: positive and negative values of bars indicate the standard deviation offset from the mean value.

Concerning the relative contribution of different PAH categories to their overall accumulation, 3- and 4-ring PAHs showed the utmost significant accumulation in site RU, followed by site UI (only for 3-ring PAHs); 5- and 6-ring PAHs significantly increased in RU samples. Two-ring PAHs did not show any significant increase in sample groups (Table 2).

Different groups of samples segregated in the ordination space defined by the first two principal components (PCs), irrespective of the fumigation treatment (Fig. 2A). Sample replicates exposed in site CK were placed at positive scores of PC 1, whereas RU and UI samples were placed at negative scores of PC 1, respectively characterized by positive and negative scores of PC 2 (Fig. 2A). Different PAH categories (3 - 6-rings) were placed in the IV quadrant of the ordination space, clearly associated to rural-urban land use, whereas 2-ring PAHs were positively associated to urban-industrial land use. Almost all of the 25 elements were placed in the 3rd and 4th quadrant: in particular, Na, Mg, V, and Sc (cluster I) were positively associated to rural-urban land use, whereas elements such as Fe, Pb and Zn (cluster II) were mostly associated to urban-industrial land use (Fig. 1, Fig. 2B).

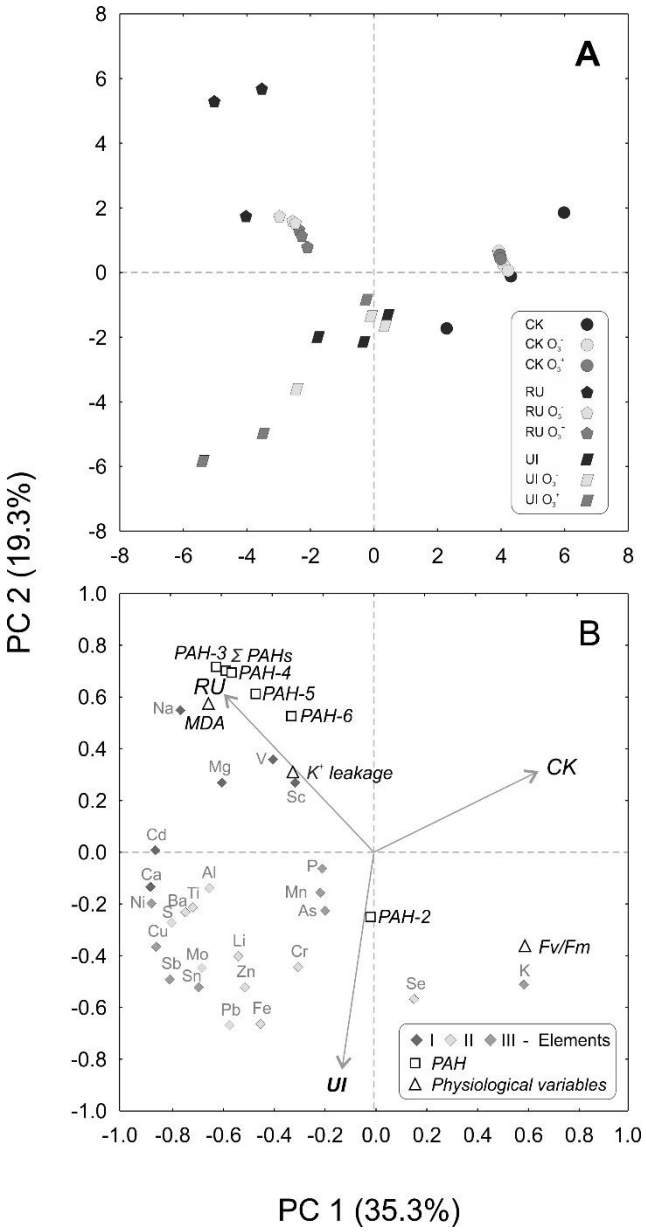


Figure 2. (A) PCA plot showing factorial scores of replicates of samples groups of the epiphytic lichen *Pseudevernia furfuracea* var. *furfuracea*, labelled as in Table 1, and (B) loading vectors of PAHs, elements, physiological variables, and their relationships with exposure site type (CK, Classical Karst; RU, rural-urban; UI, urban-industrial) plotted as supplementary variables.

3.2 Effects of field exposure and O_3-fumigation on lichen physiology

The field sites were characterized by different meteoclimatic conditions during the 6 weeks of exposure. Expectedly, the proximate-natural site (CK) was characterized by the lowest daily temperature and the highest relative humidity, whereas the opposite situation was highlighted at the urban-industrial site (UI), close to the sea. Concerning the daily temperature, site RU was more similar to site CK than to site UI, whereas the relative humidity at RU was intermediate between than of sites CK and UI (Supplementary Fig. S3 A).

After field exposure, MDA content showed the uttermost variations with respect to pre-exposure (Table 2). Such physiological pattern was also highlighted by the PCA: the MDA content showed the highest negative correlation to PC 1 (-0.65) and positive to PC 2 (+0.58). Especially, MDA was clearly associated to increasing content of PAHs, in turn associated to the rural-urban land use of

the exposure site (Fig. 2B). With respect to MDA, K^+ leakage showed a similar correlation pattern with the first two PCs, although with lower correlations in absolute figures with both axes (-0.32 with PC 1, and +0.29 with PC 2). F_v/F_m showed the opposite pattern, being positively correlated to PC 1 (+0.59) and negatively to PC 2 (-0.36), very close to the K content in the ordination space (Fig. 2B).

The CK samples showed unchanged values of MDA content, K^+ leakage and F_v/F_m with respect to BG samples (Table 2). By contrast, RU samples exhibited a significant increase of MDA content (+254.7%) and decrease of F_v/F_m (-29.8%), associated to an unchanged K^+ leakage. Finally, the UI samples exhibited a pattern similar to that of RU ones, although the physiological impairment was less pronounced (significant variation of MDA and F_v/F_m: +54.1% and -17.5%, respectively; Table 2).

The physiological parameters in fumigated samples showed a heterogeneous pattern of variation with respect to field-exposed samples. Their MDA levels significantly increased with respect to field-exposed samples for CK O_3^-, CK O_3^+ and RU O_3^- (+72.8% +134.4% and +26.2%, respectively). By contrast, UI O_3^+ samples showed a non-significant decrease in MDA content. Also, F_v/F_m levels significantly increased for RU O_3^+ (+27.9%). Finally, the K^+ leakage remained unchanged, independently of O_3 treatment (Table 2).

When focusing on matched paired samples subjected to the fumigation treatment (O_3^+ and O_3^- samples), the magnitude and sign of the differences between physiological parameters were not conserved across different experimental groups. Limiting the description to the significant variations, CK O_3^+ samples showed higher levels of MDA than CK O_3^- ones (+35.6%), whereas the opposite was found for RU O_3^+ samples (-19.5%; Table 2, Fig. 3). Such pattern was in accordance with the outcome of GLMs. Indeed, GLM results showed that, amongst the tested markers, only MDA content was significantly affected by both the field exposure and the fumigation (Table 3). In addition, the content of potassium and phosphorus (*i.e.*, K and P in Fig. 3, major elements related to the physiological status of lichen thalli) was significantly affected not only by the field exposure, but also by the fumigation treatment and their interaction (Supplementary Table S2). When tested for significant differences using Wilcoxon's matched pair test, the content of K and P was significantly lower in UI O_3^+ samples than in matched paired UI O_3^- ones. The observed pattern for K content well agrees with that observed for K^+ leakage (see *supra*).

Table 3. Summary of Generalized Linear Model (GLM) testing for main and interaction effects of field exposure (*Exp.*) and fumigation (*Fum.*) on the physiological variables (malondialdehyde content, MDA; K^+ leakage; maximum quantum efficiency of photosystem II, F_v/F_m) and the content of 4-, 5- and 6-ring PAHs measured in samples of the epiphytic lichen *Pseudevernia furfuracea* var. *furfuracea*. F-statistics and *p*-values are reported for each factor and their interaction (*p*-values < 0.05 are reported in italic). The explained variance and statistical significance of the whole model are reported as adjusted r^2 and associated significance level below the variable name.

Physiological variables				***PAHs***							
Variable	**Effect**	**F**	***p*-value**	**Variable**	**Effect**	**F**	***p*-value**	**Variable**	**Effect**	**F**	***p*-value**
MDA	*Exp.*	435.543	*< 10⁻⁴*	**Σ PAHs**	*Exp.*	239.947	*< 10⁻⁴*	**PAH-4**	*Exp.*	220.317	*< 10⁻⁴*
r^2 = 0.98	*Fum.*	5.398	*0.039*	r^2 = 0.97	*Fum.*	4.936	*0.046*	r^2 = 0.97	*Fum.*	12.267	*0.004*
p < 0.001	*Exp.×Fum.*	25.312	*< 10⁻⁴*	p *< 0.001*	*Exp.×Fum.*	6.037	*0.015*	p *< 0.001*	*Exp.×Fum.*	17.438	*2.8 × 10⁻⁴*
K^+ leakage	*Exp.*	1.734	0.218	**PAH-2**	*Exp.*	3.016	0.087	**PAH-5**	*Exp.*	2.262	0.147
r^2 = 0.03	*Fum.*	0.240	0.633	r^2 = 0.06	*Fum.*	0.001	0.974	r^2 = 0.11	*Fum.*	1.357	0.267
p > 0.05	*Exp.×Fum.*	0.869	0.444	p > 0.05	*Exp.×Fum.*	0.063	0.939	p > 0.05	*Exp.×Fum.*	0.614	0.557
F_v/F_m	*Exp.*	2.353	0.137	**PAH-3**	*Exp.*	289.870	*< 10⁻⁴*	**PAH-6**	*Exp.*	0.995	0.398
r^2 = 0.07	*Fum.*	0.126	0.728	r^2 = 0.97	*Fum.*	0.0491	0.828	r^2 = -0.21	*Fum.*	0.014	0.909
p > 0.05	*Exp.×Fum.*	0.764	0.487	p *< 0.001*	*Exp.×Fum.*	0.0428	0.958	p > 0.05	*Exp.×Fum.*	0.023	0.978

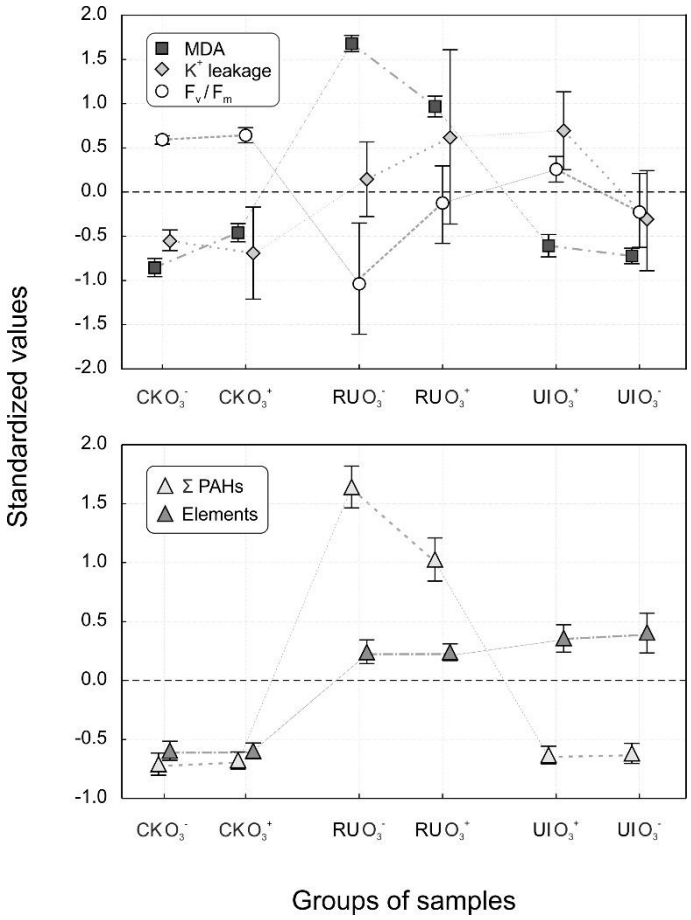


Figure 3. Standardized values of physiological variables (top of the figure), overall PAH and elemental content (bottom of the figure). Different groups of samples of the epiphytic lichen *Pseudevernia furfuracea* var. *furfuracea* are labelled as in Table 1. Data are shown as means and 95% confidence interval (positive and negative values of bars indicate the standard deviation offset from the mean value).

4. Discussion

4.1 A rather tolerant biomonitor

In this work, we evaluated the physiological response of *Pseudevernia furfuracea* var. *furfuracea* to a multi-pollutant field and laboratory exposure. The lichen physiological status was evaluated at each experimental step in terms of photosynthetic activity of the algal population (*i.e.*, F_v/F_m), peroxidation of membrane lipids (*i.e.*, MDA), and membrane integrity (*i.e.*, K^+ leakage).

Lichens collected in the BG area showed Chl_aF values fully consistent with those previously assessed for the same species. Indeed, F_v/F_m values lied within the range reported by several studies for unstressed *P. furfuracea* thalli (0.610 - 0.750: *e.g.*, Calatayud et al. 1997; Niewiadomska et al. 1998; Vidergar-Gorjup et al. 2001; Tretiach et al. 2007; Malaspina et al. 2018). Even for MDA our BG values were consistent with the few data available for *P. furfuracea* from background sites (*c.* 10 nmol g^{-1} DW: Corapi et al. 2014; Lucadamo et al. 2015), after correction of measurement units (*in litt.* to Lucadamo). Concerning K^+ leakage, reference values for *P. furfuracea* are missing; however, our values were lower than those reported for unstressed samples of the trebouxioid lichen *Flavoparmelia caperata* (0.170 - 0.300 mg g^{-1} DW; Candotto Carniel et al. 2017).

Overall, the physiological impairment of *P. furfuracea* after the field exposure was rather limited, although sometimes significant. Indeed, significant variations were highlighted for lipid

peroxidation and chlorophyll fluorescence in samples exposed at the rural-urban site (RU), where PAH depositions were the highest (Fig. 1). Instead, the O_3-treatment produced a significant increase of MDA limited to samples previously exposed in the semi-natural site of the Classical Karst (*i.e.*, CK O_3^+ samples) (Table 2; Fig. 3). Our results showed that, amongst the tested physiological markers, MDA and F_v/F_m showed the clearest response to pollution and environmental changes (Fig. 2). K^+ leakage was previously used as a very effective biomarker of SO_2 (Tarhanen et al. 1996) and trace element pollution (Tarhanen et al. 1999; Cuny et al. 2002); however, in our case, K^+ leakage showed generally low mean values, and a non-significant pattern of variation (Table 2), as well as non-significant correlations with targeted elements (ranging from -0.26 to 0.40) and other markers (0.37 and -0.42 with MDA and F_v/F_m, respectively). On the one hand, this result suggests that SO_2 and metal pollution experienced by the exposed thalli was relatively low and, on the other hand, supports the idea that K^+ leakage may not uniquely reflect the loss of membrane integrity caused by lipid peroxidation, as previously hypothesized (Cuny et al. 2004). The F_v/F_m values after the field exposure were fully consistent with those reported by other authors for the same species (Tretiach et al. 2007; Malaspina et al. 2018). Indeed, the decrease of F_v/F_m was rather limited, confirming the good resistance of this lichen to the typical urban environmental conditions (such as high temperatures and irradiance, and low air humidity). Undoubtedly, this typical light-demanding lichen of exposed environments (Wirth 1995) owns specific physiological and morpho-anatomical features which also allow it to cope with prolonged desiccation (Rikkinen 1997).

When interpreting the physiological pattern of experimental samples, attention should be paid on the effects of possible confounders. As a matter of fact, the physiological response of lichens transplanted to urban environments is influenced by several factors, especially by the interplay of climatic conditions (see Supplementary Methods S1 for a climatic description of the exposure area) and air pollutants (Tretiach et al. 2012). For these reasons, monitoring meteoclimatic conditions, as well as the phytotoxic pollution loads during the exposure of lichen samples, is of primary importance (Piccotto et al. 2011). As far as meteoclimatic conditions are concerned, the sites in the Classical Karst (CK and RU) experienced higher rainfall than the UI site during the 6 weeks exposure (125 *vs* 96 mm; OSMER FVG 2018), well reflecting climatic differences between these contexts (Supplementary Methods S1). The wind regime during the exposure period clarifies the generally low enrichment levels revealed in samples exposed at the UI site. The prevailing Bora wind, blowing from northeast towards the sea, definitely limits the lichen enrichment related to particulate matter depositions. Indeed, only Na and Fe were heavily accumulated (Sect. 3.1), respectively reflecting the sea influence (all exposure sites) and the nearby presence of a large cast-ironwork (site UI) (Supplementary Fig. S1).

The more favourable conditions at CK and RU sites (see Sect. 3.2 and Supplementary Fig. S3 A) suggest that the meteoclimatic gradient alone cannot explain the observed physiological pattern, since the highest detrimental effects were observed in lichens transplanted at site RU, meaning that pollutant gradients must be definitely considered as prominent. In this respect, we cannot exclude an effect of phytotoxic gaseous pollutants such as nitrogen oxides (NO_x) and SO_2, which are known to cause an impairment of the maximum quantum yield of primary lichen photochemistry (Rao and LeBlanc 1966; Beckett et al. 2008; Piccotto et al. 2011). During the field exposure, the mean hourly concentrations of NO_2 and SO_2 measured at a monitoring station near the UI site were 32.0 ± 21.0

and 2.1 ± 3.4 µg m^{-3} (ARPA FVG 2018b, 2018c; Supplementary Fig. S3 B), with maximum values far lower than the EU limits for human health (EU Directive 2008/50/EC). It is not possible to fully disentangle the physiological effects due to different pollutants during the field exposure of lichen samples; however, NO_2 emissions in urban environments are mainly related to vehicular traffic (definitely prominent in UI site), whereas wood burning activities, characterizing site RU, are acknowledged as sources of SO_2 (Cooper 1980). Consequently, these sites can rightly be considered as affected by the highest winter levels of NO_2 and SO_2, respectively. Therefore, a combination of metal-rich particulate matter and NO_x plausibly caused the (rather limited) physiological impairment observed in samples exposed at site UI, whereas a combination of high PAH levels and SO_2 caused that of samples exposed at site RU, including the significant increase of MDA levels. However, in this respect, the strong match between the trends of PAHs and MDA content suggests a prominent role of PAHs with respect to SO_2 (Fig. 2; Fig. 3).

After the fumigation, the heterogeneous physiological pattern of samples (Sect. 3.2) depicted an interesting scenario. Contrarily to our original hypothesis, the CK samples, that experienced the lowest pollution and the most favourable environmental conditions during the field exposure, suffered in relative terms the highest damage, reflected by a significant increase in MDA content affecting both CK O_3^+ and CK O_3^- samples. Considered the good health status of these samples immediately after the field exposure, it is highly feasible that these suffered a so-called "chamber effect" related to the regime of steady temperatures in the fumigation chambers (Bertuzzi et al. 2013), which were higher than those in the field (Sect. 2.3, Supplementary Fig. S3 A). Nevertheless, this was not enough to damage the algal population, because the Chl$_a$F levels remained stable (Table 2).

Intriguingly, for UI samples (which experienced more stressing conditions than CK samples) a recovery of the algal population was observed, independently of O_3 treatment. Although variations were not significant, F_v/F_m increased by 15% and 8% in O_3^- and O_3^+ samples with respect to field-exposed ones. F_v/F_m values also exhibited slight differences between UI O_3^+ and UI O_3^- samples, the latter having value 6% higher than the former (Table 2). The fluorescence pattern of O_3-treated CK and UI samples well matches with the results of the other single work that addressed the physiological response of *P. furfuracea* to O_3 (Scheidegger and Schroeter 1995). In accordance with our results, *P. furfuracea*, exposed for 80 days to 180 µg m^{-3} (day) and 80 µg m^{-3} (night) of O_3 (*c.* 90 and 40 ppb) in field fumigation chambers, always showed non-significant variation of F_v/F_m values (Scheidegger and Schroeter 1995). This pattern is also consistent to that highlighted for other trebouxioid lichen species (Riddell et al. 2010; Pellegrini et al. 2014; Bertuzzi et al. 2018). However, an overall algal recovery occurred for fumigated UI samples. *P. furfuracea* is a desiccation tolerant species and a photobiont recovery was previously observed in fully controlled environment after a stressing field exposure (*e.g.*, Kranner et al. 2003). Thus, since we purposely avoided the exposure of wet thalli to relatively high light during their stay in the fumigation chambers (Bertuzzi et al. 2013; Pellegrini et al. 2014), the observed pattern contravenes our original hypothesis.

RU samples also exhibited an overall recovery of the algal population during their stay in the fumigation chambers, with F_v/F_m increased by 13% and 28% in O_3^- and O_3^+ samples with respect to field-exposed ones. In this case, the same considerations spelt out for UI samples apply, hence

definitely proving the noteworthy O_3-tolerance of *P. furfuracea*. By contrast, the physiological pattern of F_v/F_m and MDA content in matched fumigated RU samples is opposite to that of UI samples. Indeed, RU O_3^- samples exhibited higher MDA content and lower F_v/F_m than RU O_3^+ ones (differences that were respectively significant or not; Sect. 3.2). Most likely, such pattern has to be interpreted in relation to possible interaction phenomena between high levels of PAHs accumulated during the field exposure and the O_3 subsequently provided, which deserves further explanation.

4.2 Does summer-like ambient air O_3 affect PAH content in lichen biomonitors?

An intriguing aspect, with possible important implications for the interpretation of biomonitoring results, concerns the possible interactions between O_3 and Polycyclic Aromatic Hydrocarbons (PAHs). Indeed, the ability of O_3 in degrading either adsorbed or gas phase PAHs has repeatedly been demonstrated in different environmental matrices (Yao and Masten 1992; Nam and Kukor 2000) such as soils, sediments, water and sludges (*e.g.*, Kochany and Maguire 1994; Bernal-Martinez et al. 2005; Haapea and Tuhkanen 2006; Hong et al. 2008). Recently, Kodnik et al. (2015) hypothesized that such oxidative degradation reactions could also occur at thallus level in lichen transplants. In this respect we found an interesting pattern in relation to fumigated samples that experienced the highest field loads of PAHs.

Firstly, the content of PAHs in our lichen samples was generally lowered after their 2 week-stay in the fumigation chambers, especially in samples previously exposed at sites RU and UI, and such loss was spread amongst O_3^+ and O_3^- samples. Since all samples were carefully stored at the same conditions according to standardized procedures (Augusto et al. 2013) and contemporarily analysed, this loss (positively related to the level of PAH bioaccumulation occurred in the field) has most likely occurred during the fumigation. Interestingly, it was recently demonstrated that gas phase Fl and B[a]Py accumulate in the photosynthetic algal layer, but no major losses were observed during their stay in climatic chambers (Augusto et al. 2015). However, no results are available for other aromatic compounds and PAHs adsorbed to particulate matter. Since particulate is mainly deposited onto the lichen surface or trapped in the intercellular spaces of the medulla (Garty et al. 1979), it does make sense to assume that, depending on the compound-specific physicochemical characteristics, as well as on the environmental conditions and residence time in controlled environment, a loss of PAHs from lichen thalli could occur. Moreover, although PAHs have low solubility in water (Huang et al. 1993), it is also possible that these could be mechanically washed off due to daily rehydration procedures (Sect. 2.2), as also expected by Augusto et al. (2015), but contextually not observed by the authors (Augusto et al. 2015).

Having said that, a clear and noticeable pattern emerged for RU O_3^+ and RU O_3^- samples previously exposed in a site where the wood burning in traditional fireplaces is the prevailing source of aerodisperse organic compounds (Mastral and Callén 2000; Kodnik et al. 2015). Accordingly, these were characterized by far by the utmost content of Fl, Py, B[a]Ant and Chry (4-ring PAHs), the typical tracers of wood burning activities (Boström et al. 2002; Singh et al. 2013) (Fig. 4; Supplementary Fig. S4). Interestingly, after fumigation, F_v/F_m in RU O_3^+ samples was higher than in RU O_3^- ones, whereas the MDA and the 4-ring PAH content was significantly lower. In particular, the content of Fl, Py, Chry and B[a]Ant in RU O_3^+ samples was 23%, 46%, 86% and 89% lower than in the paired RU O_3^- samples (Wilcoxon matched paired test, $p < 0.05$;

Supplementary Fig. S4). This suggests that ozonation caused the degradation of PAHs with subsequent physiological improvement.

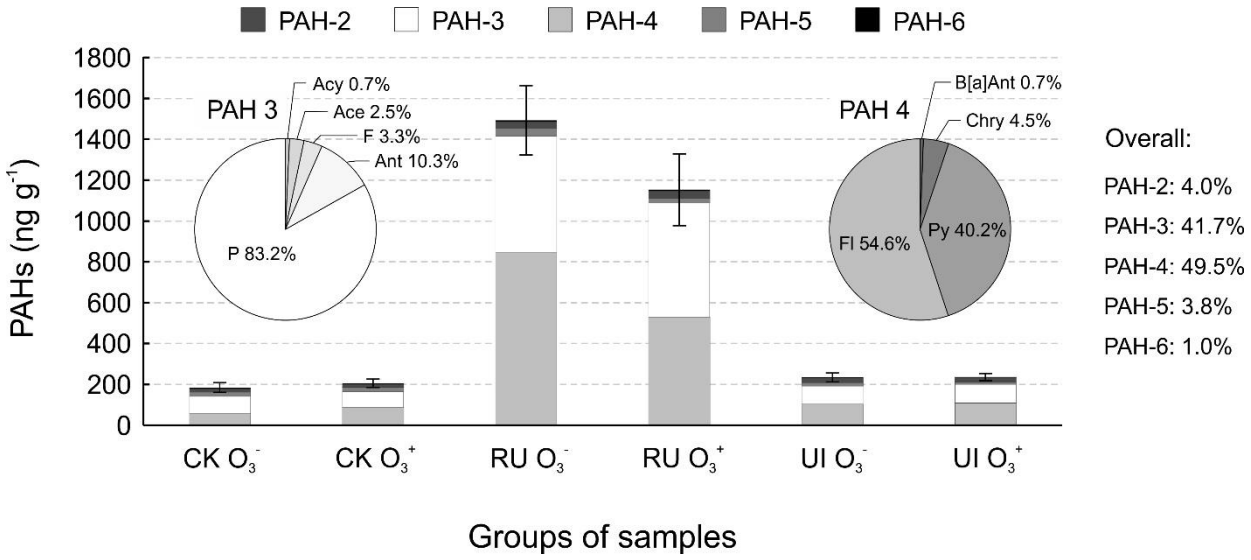


Figure 4. PAH content in exposed thalli of the epiphytic lichen *Pseudevernia furfuracea* var. *furfuracea*, either ozonated or not (groups of samples are labelled as in Table 1). Data refer to mean values of 2-, 3-, 4-, 5- and 6-ring PAHs and overall standard deviations. The pie chart insets show the contribution of 3- and 4-ring PAHs, the most abundant polycyclic aromatic compounds revealed in exposed samples.

The effectiveness of O_3 in degrading PAHs depends on the targeted matrix (Von Gunten 2003; Masten and Davies 1997; Goi and Trapido 2004), the molecular weight and structure of the compounds, their physical state (Abdel-Shafy and Mansour 2016) and oxidant doses (Siegrist et al. 2011). The structural and chemical complexity of the lichen matrix does not allow conclusive statements, and the experimental design was not conceived to test this specific hypothesis. Nonetheless, in light of such overt pattern affecting matched fumigated RU samples, it is highly feasible that O_3 treatment acted as a depletion agent for the most abundant residual PAHs retained at the algal layer. Indeed, lichens lack the waxy cuticle that characterizes the leaves of vascular plants, hence permitting the diffusion of O_3 towards the inner lichen layers (Pellegrini et al. 2014). It could be argued why a similar depletion pattern was not observed for the second most abundant class of the 3-ring PAHs. In this regard, it is known that the O_3 treatment of 4-ring PAHs generally produces 3-ring by-products (Cochran et al. 2016), that, according to their fate, may mask a lowering trend, hence misleading the interpretation of results due to possible chromatographic interferences with native PAHs (Janska et al. 2006; Fromberg et al. 2007). Undoubtedly, further research is needed to clarify the fate of different phase PAHs in lichens, however, the monitoring of O_3 ground levels should become a routine precaution during summer campaigns with lichens as biomonitors, in order to avert potential underestimation of bioaccumulated PAHs.

5. Conclusions

This study provides another piece to the lichen O_3-tolerance puzzle faced in a number of recent studies (Riddell et al. 2010; Bertuzzi et al. 2013, 2018; Pellegrini et al. 2014). In particular, *Pseudevernia furfuracea* var. *furfuracea* stands as a rather tolerant lichen biomonitor, confirming its

ability to cope with typical urban environmental conditions. A physiological impairment, mostly highlighted in terms of a significant increase in MDA levels, was caused by massive PAH loads (and possibly by SO_2) during the field exposure at wood-burning chimneys at the RU site. However, *P. furfuracea* did not experience major detrimental effects due to the O_3 treatment. In particular, samples that experienced more stressing field conditions, exhibited a recovery of the algal populations or, at most, no significant variations of other markers. This result contravenes our original hypothesis and highlights that the test species can be classified as fully tolerant to ozone.

Furthermore, limited to samples exposed to high PAH loads, the peculiar physiological and PAH pattern affecting fumigated samples suggested that a significant decrease of PAHs in ozonated samples was possibly ascribable to oxidative degradation occurring at the thallus system level, specifically consisting in the degradation of the most abundant 4-ring PAHs accumulated during the field exposure. Although further investigation is needed to clarify the issue, the possibility of an underestimation of PAH enrichment levels should seriously be considered when carrying out transplant-based surveys with contextually high O_3 ground levels.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS S1

S1.1 Climatic characterization of the exposure area

The exposure area is characterized by a fully humid, warm temperate climate with hot summers on the coast and warm ones in the interior (Kottek et al. 2006), being transitional between sub-Mediterranean and pre-Alpine climates. In particular, the Classical Karst is characterized by higher annual rainfall than the city of Trieste (1341 *vs* 1015 mm yr^{-1}; Stravisi 2010). The entire area is subjected to marked seasonal changes (Nimis 1982; OSMER FVG 2018) and to predominant continental dry winds blowing from ENE ("Bora"), particularly frequent between October and April (approximately 80 days yr^{-1} in Trieste) (Furlani et al. 2009; Stravisi 2010). Warm-humid winds blowing from SW ("Scirocco") normally bring rainy weather, so that the alternation of Bora and Scirocco characterizes the winter climate of the entire area (Nimis 1982).

SUPPLEMENTARY TABLES S1-S2

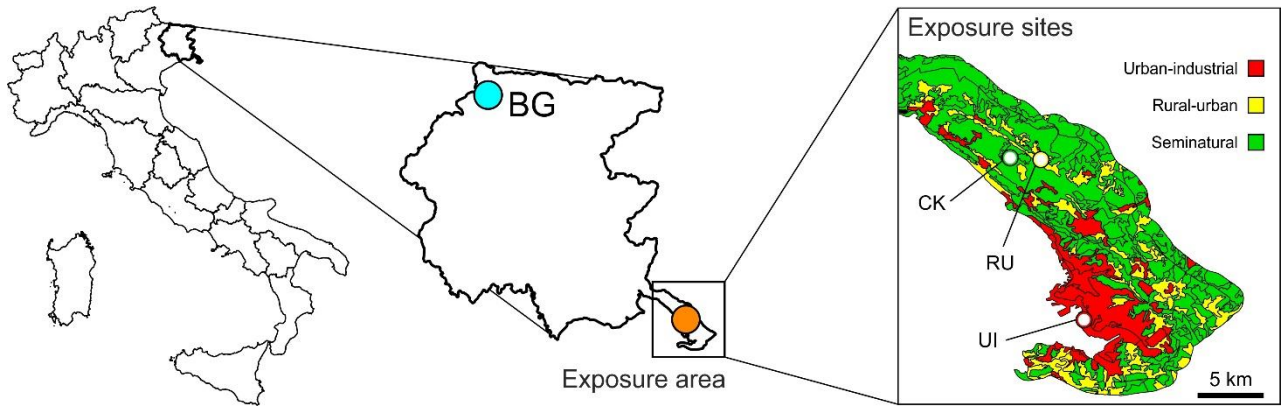
Supplementary Table S1. Mean recovery percentages and 95% confidence interval (95% C.I.) of element content in the standard reference material BCR 482 'lichen' *Pseudevernia furfuracea*. Mean recoveries and confidence interval are calculated on 4 analytical replicates.

Element	BCR 482 recovery (95% C.I.)
Al	95.2 (86.9 ÷ 103.5)
As	100 (-11.8 ÷ 211.8)
Ba	82.2 (76.9 ÷ 87.6)
Ca	76.2 (71.3 ÷ 81.2)
Cd	96 (83.6 ÷ 108.3)
Co	93.8 (93.8 ÷ 93.8)
Cr	103.2 (83.8 ÷ 122.5)
Cu	102.1 (98.7 ÷ 105.5)
Fe	96.4 (86.5 ÷ 106.3)
Mn	85.6 (76.5 ÷ 94.7)
Ni	99.2 (90.9 ÷ 107.5)
Pb	86.3 (82.3 ÷ 90.2)
V	86.9 (65.6 ÷ 108.2)
Zn	122.6 (59.3 ÷ 185.8)

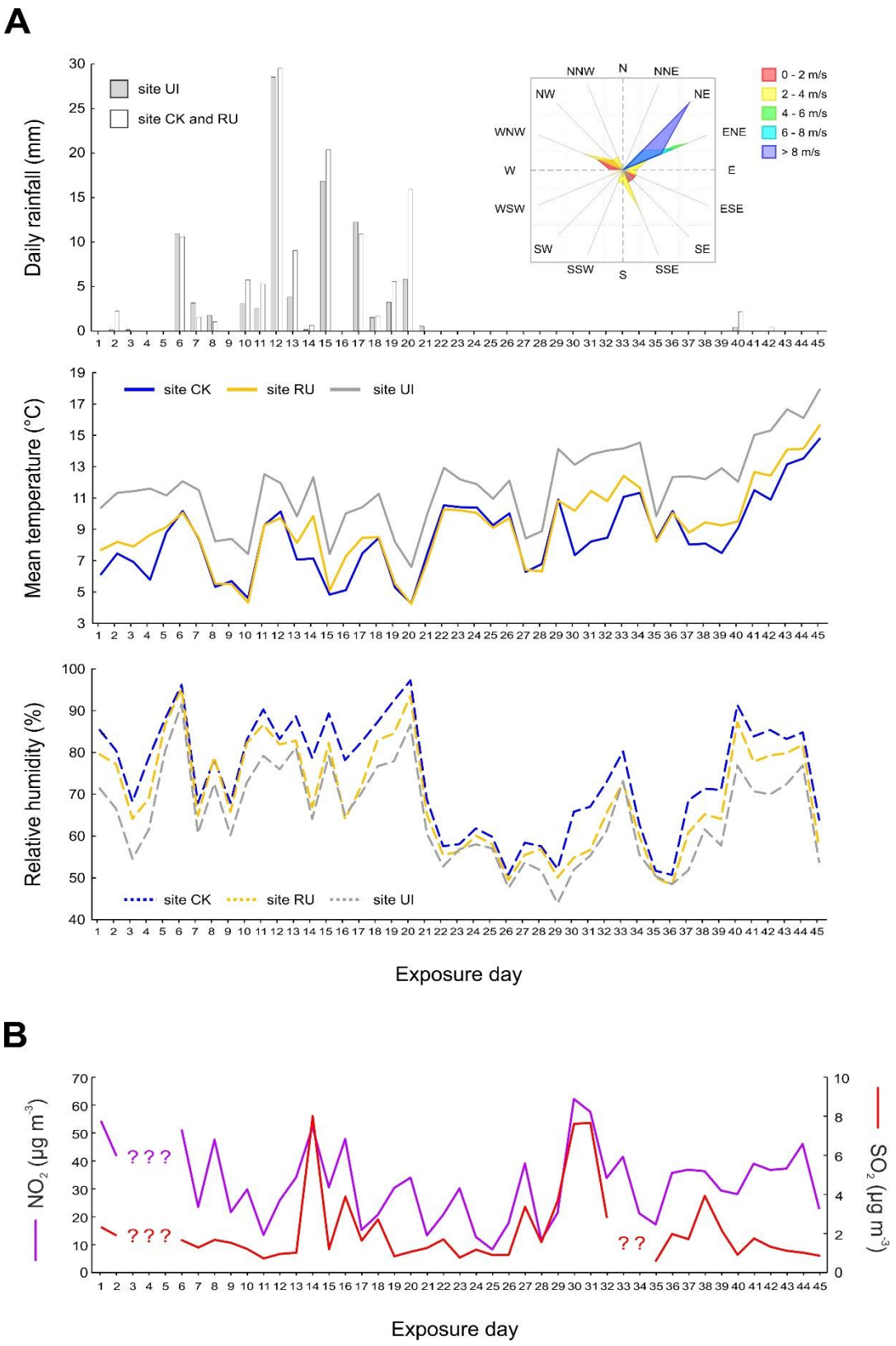
Supplementary Table S2. Summary of Generalized Linear Model (GLM) testing for main and interaction effects of the field exposure (*Exp.*) and ozonation (*Fum.*) on the content of 25 chemical elements (μg g^{-1}) measured in samples of the epiphytic lichen *Pseudevernia furfuracea* var. *furfuracea*. F-statistics and *p*-values are reported for each factor and their interaction (*p*-values < 0.05 are reported in italic). The explained variance and statistical significance of the whole model are reported as adjusted r^2 and associated significance level (* $0.05 < p \leq 0.01$; ** $0.01 < p \leq 0.001$; *** $p < 0.001$).

Effect	F	*p*-value	F	*p*-value	F	*p*-value	F	*p*-value	F	*p*-value
	Al (r^2 = 0.59 **)		As (r^2 = 0.50 *)		Ba (r^2 = 0.57 **)		Ca (r^2 = 0.67 **)		Cd (r^2 = 0.61 **)	
Exp.	13.000	*0.001*	10.478	*0.002*	10.326	*0.002*	19.329	*1.8 × 10^{-4}*	15.925	*4.2 × 10^{-4}*
Fum.	1.000	0.337	0.391	0.543	2.326	0.153	0.086	0.774	0.025	0.877
Exp. × *Fum.*	1.000	0.397	0.391	0.684	2.326	0.140	0.086	0.918	0.025	0.975
	Cr (r^2 = 0.00)		Cu (r^2 = 0.35)		Fe (r^2 = 0.91 ***)		K (r^2 = 0.96 ***)		Li (r^2 = 0.58 **)	
Exp.	1.000	0.397	6.952	*0.010*	93.143	*< 10^{-4}*	159.200	*< 10^{-4}*	4.750	*0.030*
Fum.	1.000	0.337	0.023	0.883	0.143	0.721	28.800	*1.7 × 10^{-4}*	6.250	*0.028*
Exp. × *Fum.*	1.000	0.397	0.023	0.978	0.143	0.868	28.800	*< 10^{-4}*	6.250	*0.014*
	Mg (r^2 = 0.72 ***)		Mn (r^2 = -0.25)		Mo (r^2 = 0.27)		Na (r^2 = 1.00 ***)		Ni (r^2 = 0.32)	
Exp.	24.000	*< 10^{-4}*	0.385	0.689	4.192	*0.042*	1810.300	*< 10^{-4}*	6.537	*0.012*
Fum.	0.000	1.000	0.285	0.603	0.962	0.346	2.500	0.140	< 10^{-4}	1.000
Exp. × *Fum.*	0.000	1.000	0.285	0.757	0.962	0.410	2.500	0.124	< 10^{-4}	1.000
	P (r^2 = 0.65 **)		Pb (r^2 = 0.38)		S (r^2 = 0.76 ***)		Sb (r^2 = 0.69 **)		Sc (r^2 = 0.41 *)	
Exp.	6.142	*0.015*	6.385	*0.013*	6.385	*0.013*	24.800	*< 10^{-4}*	7.000	*0.010*
Fum.	7.934	*0.016*	0.880	0.367	0.880	0.367	3.200	0.099	1.000	0.337
Exp. × *Fum.*	7.934	*0.006*	0.880	0.440	0.880	0.440	3.200	0.077	1.000	0.397
	Se (r^2 = 0.62 **)		Sn (r^2 = 0.32)		Ti (r^2 = 0.04)		V (r^2 = 0.19)		Zn (r^2 = 0.25)	
Exp.	15.750	*4.4 × 10^{-4}*	3.500	0.063	2.600	0.115	3.000	0.088	4.402	*0.037*
Fum.	0.250	0.626	2.000	0.183	0.200	0.663	1.000	0.337	0.605	0.452
Exp. × *Fum.*	0.250	0.783	2.000	0.178	0.200	0.821	1.000	0.397	0.605	0.562

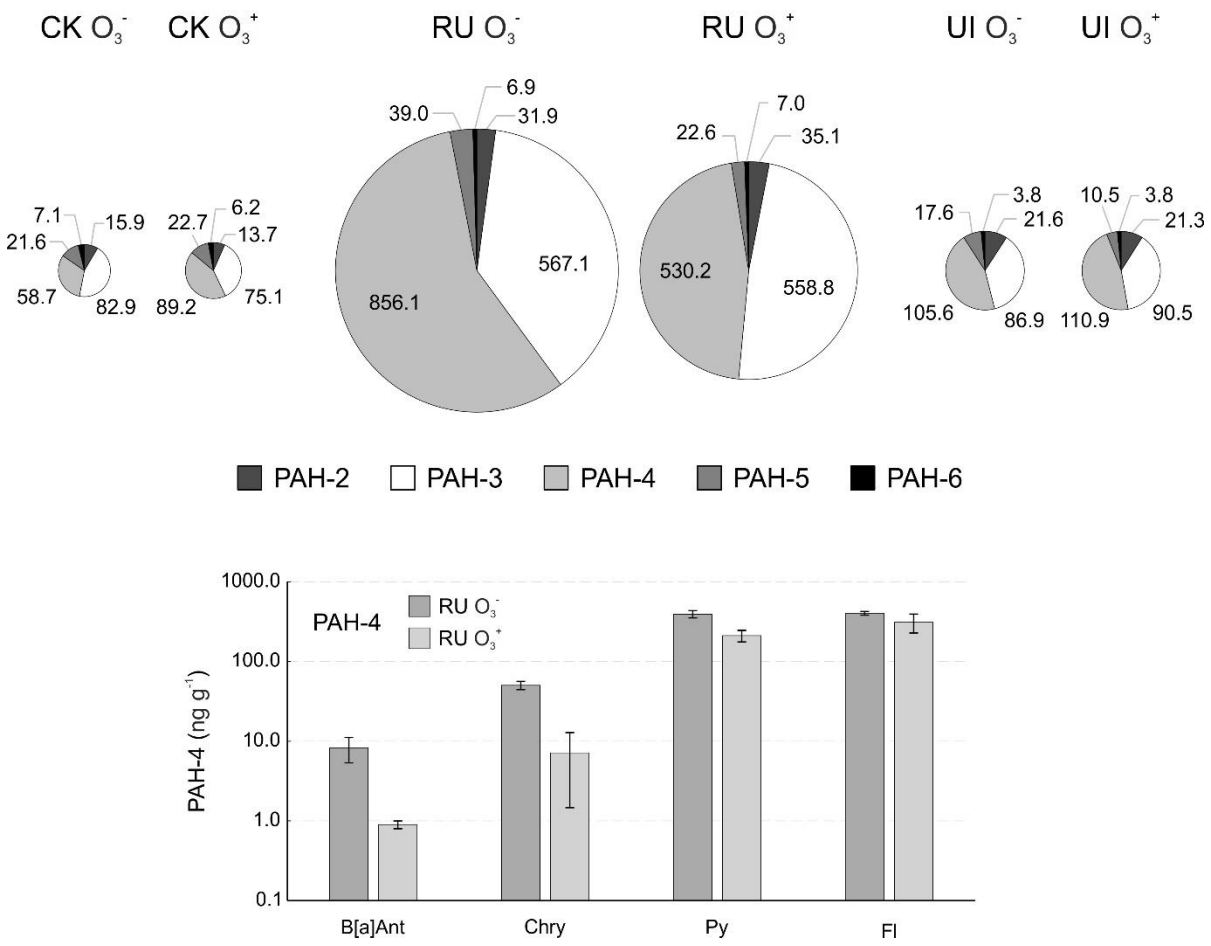
SUPPLEMENTARY FIGURES S1-S2



Supplementary Figure S1. Geographic location of the collection (BG) and the exposure sites (CK, RU, UI) of the epiphytic lichen *Pseudevernia furfuracea* var. *furfuracea* samples (NE Italy).



Supplementary Figure S2. (A) Meteorological conditions during the sample field exposure. From the top to the bottom: daily rainfalls in the urban-industrial (UI) site and in a locality of the Classical Karst close to both control and rural-urban (CK and RU) sites (the inset shows the wind direction and intensity measured in the city of Trieste, close to site UI; OSMER FVG 2018); mean daily temperature and relative humidity at the three exposure sites. (B) Daily atmospheric concentrations of phytotoxic gases (NO_2 and SO_2) measured at a monitoring station close to the UI site. Question marks highlight missing data.



Supplementary Figure S3. Specific composition of PAHs in exposed and ozonated samples of the epiphytic lichen *Pseudevernia furfuracea* var. *furfuracea* (groups of samples are labelled as in Table 1). Values refer to PAH content values expressed in ng g^{-1}, as reported in Table 2. Different areas of pie charts reflect the overall content of such compounds, represented with respect to the maximum PAH content revealed amongst different groups of samples (that of RU O_3^-).

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Element accumulation performance of living and dead lichens in a large sample-sized transplant application

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Highlights

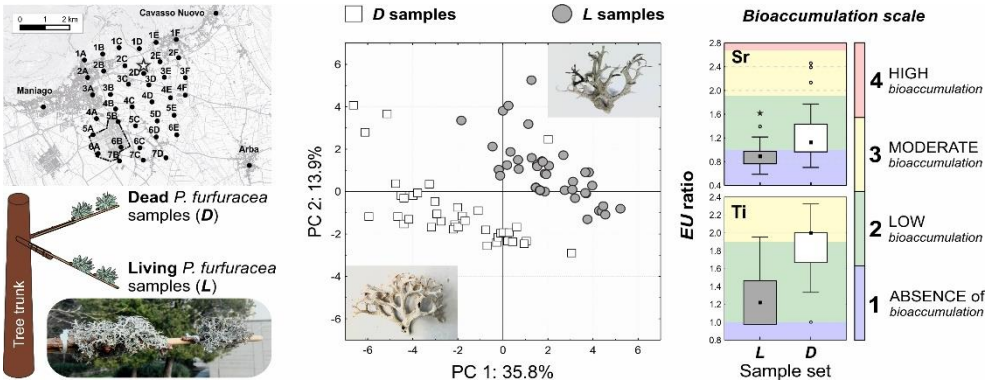
• Lichen devitalization was achieved avoiding typical physico-chemical treatments
• Dead lichens transplanted at 40 sites showed higher accumulation for most elements
• Dead samples highlighted the finest depositional patterns in the study area
• Using samples with different health states may bias result interpretation

Abstract

In bioaccumulation studies by mosses, devitalization through acid washing ("activation") or oven-drying is standardly carried out on monitoring samples, since it enhances the efficiency of contaminant capture by passive uptake processes, as highlighted by field and laboratory experiments. Such an aspect was never addressed for lichens in biomonitoring surveys of airborne trace elements. In this study, the trace element accumulation performance of living (*L*) and dead (*D*) samples of the lichen *Pseudevernia furfuracea* is assessed through a large sample-sized transplant study. Devitalization was achieved by a long-term storage in a dark-cool room, in order to test the hypothesis that an enhanced bioaccumulation signal may occur without carrying out physico-chemical treatments on the lichen matrix. Paired *L-D* samples were exposed for 8 weeks at 40 sites in a mixed land use area of NE Italy. Before and after exposure, the health status of lichen samples was assessed by chlorophyll fluorescence emission.

The two sample sets consistently described generally low pollutant depositions over the study area. However, significantly higher accumulation signals were revealed in dead lichens for Al, Ca, Fe, Ti, As, Cd, Co, Cu, Hg, Pb, Sr, Zn. This leaded to some interpretational discrepancies when the element concentrations of the two sample sets were classified according to the new Bioaccumulation Scale for lichen transplants.

Dead lichens demonstrated to be able to highlight the finest depositional patterns in a composite area. In this light, the possibility of using dead lichen samples in biomonitoring surveys should be considered to contribute to the methodological standardization and harmonization of the lichen transplant technique.



Keywords: bioaccumulation, active biomonitoring, *Pseudevernia furfuracea*, interpretative scale

1. Introduction

Lichens and mosses are highly performing bioaccumulators, which provide reliable information on the source apportioning of airborne elements and their depositional patterns (Giordano et al. 2013). For this reason, their use is frequently recommended as complementary to conventional monitoring by instrumental devices (Marć et al. 2015).

The wide application of biomonitoring techniques by lichens and mosses over years triggered a major research interest for the processes underlying metal accumulation (e.g., Garty et al. 1979; Brown and Beckett 1985; Tyler 1989; Vázquez et al. 1999). These processes may be very complex, as many factors affect the element accumulation by biological systems (or even by their individual symbionts, in case of symbiotic organisms; Bačkor and Loppi, 2009). However, in spite of such interest and the growing supportive role of biomonitoring in environmental forensics and decision-making processes, the research aimed at enhancing the methodological consistency of biomonitoring techniques has often followed separated pathways for mosses and lichens. This produced unbalanced outcomes in terms of available protocols, supra-regional sampling networks, data quality and comparability (Cecconi et al. 2019a). A perfect illustration of this phenomenon is represented by the investigation of trace element bioaccumulation in relation to the vitality of the biomonitor. As a matter of fact, such an aspect was frequently addressed in the framework of active "bryomonitoring" (i.e., biomonitoring by the *moss bag* technique; e.g., Aničić et al. 2009a, 2009b; Basile et al. 2009; Giordano et al. 2009; Deben et al. 2016), whereas it has scarcely been faced for lichens.

To date, it is acknowledged that devitalization of moss gametophytes enables an enhanced efficiency of contaminant capture by passive uptake processes (see Ares et al. 2012 and references therein). Especially, the particulate interception and entrapment at the surface level is enhanced in dead mosses (Giordano et al. 2013), with useful effects in terms of achievable trace element pollution signals. Further advantages of devitalizing samples would consist in the reduced variability of results at site level due to the absence of (*i*) metabolic activity (Giordano et al. 2009; Capozzi et al. 2017), and (*ii*) growth during the exposure period (which is a non-negligible source of data variability; Fernández et al. 2009; Fortuna and Tretiach 2018). In this light, the leitmotif of sample devitalization has been carried forward with great consistence in the bosom of bryomonitoring, as reflected by the 'Mossphere', a highly standardized exposure device of recent development which uses devitalized shoots of an axenically cultured *Sphagnum palustre* clone (Reski et al. 2016).

Differently, the influence of lichen vitality on the efficiency of elemental accumulation was addressed in a single field work. Indeed, Adamo et al. (2007) assessed the accumulation performance of the macrolichen *Pseudevernia furfuracea* (L.) Zopf in comparison to that of the moss *Hypnum cupressiforme* Hedw. in a 6-week transplant experiment carried out in two Italian sites with different pollutant loads and climatic conditions. Besides performing an inter-species comparison, the authors demonstrated that living *P. furfuracea* samples did not show a better performance with respect to dead ones (Adamo et al. 2007).

Irrespective the test species, in most methodological studies targeting the issue of biomonitor vitality in relation to bioaccumulation, devitalization is generally carried out by acid washings

and/or oven-drying (Ares et al. 2012). Acid washing (or "activation") consists in rinsing the material in an acid medium, with the aim of leaching metal ions from the cell walls and disrupting biological membranes, hence regenerating the cation exchange sites to increase the bioconcentration capacity (Brown and Wells, 1988; Brown and Brown, 1991; Adamo et al. 2007). This procedure notably deteriorates the tissues (Giordano et al. 2009). In oven-drying, the material is simply maintained at temperatures higher than 100 °C for 24 h, thus it possibly causes the volatilization of some elements (Ares et al. 2012). Oven-drying alters much less the morphological structure of biomonitors, also being eco-friendlier than acid washing (Giordano et al. 2009).

Another aspect common to these studies is that the accumulation efficiency of living and dead biomonitors is generally tested by transplanting paired living-dead samples at a little number of sites (e.g., Adamo et al. 2007; Giordano et al. 2009; Debén et al. 2016). Therefore, although the experimental design provides with a discrete number of replicates, poor conclusions can be drawn on the potential interpretational bias resulting from the exposure of samples with different health status in a real, large sample-sized survey.

In this work, the hypothesis that living and dead lichen matrices differ in terms of accumulation efficiency is tested using the highly performing lichen bioaccumulator *P. furfuracea*, the only species for which this issue was previously addressed, therefore providing a starting point to perform reliable result comparison. The choice of the species is also dictated by its widespread use in active biomonitoring (e.g., Adamo et al. 2003; Jozic et al. 2009; Tretiach et al. 2011; Petrova et al. 2015) and its role in methodological studies (e.g., Incerti et al. 2017; Cecconi et al. 2018; Cecconi et al. 2019b, 2019c), that has led to the development of the very last interpretative tool for lichen bioaccumulation data from transplant applications (Cecconi et al. 2019a). Here, for the first time, the issue of *P. furfuracea* vitality in relation to its accumulation capacity is faced in a large sample-sized transplant application carried out in an area of NE Italy, already used in methodological studies (Kodnik et al. 2015, 2017). Devitalization of lichens was achieved by a long-term storage in a dark-cool room, in order to (*i*) avoid the alteration of the initial chemical composition of samples, as possibly caused by more aggressive procedures (Adamo et al. 2007, 2008), and (*ii*) to test the hypothesis that an enhanced bioaccumulation signal may also occur without carrying out physico-chemical treatments on lichen matrices. Moreover, the potential interpretative bias of bioaccumulation results due to the use of dead vs. healthy lichens is addressed, as a further contribution to the discussion on the standardization of biomonitoring techniques based on terrestrial cryptogams.

2. Materials and methods

2.1 Lichen collection, sample pre-treatment and storage

On December 8th 2016, c. 400 thalli of *Pseudevernia furfuracea* were collected in an acknowledged background area of the Carnic Alps (317614 E, 5148046 N; 1750 m a.s.l.; Cecconi et al. 2018, 2019b).

After the cleaning and selection procedures (for details see Cecconi et al. 2019b), the bulk material was split into two sets subjected to different storage conditions. A half of thalli was air dried, vacuum-sealed and stored in freezer at -20 °C in order to preserve their vitality (Honegger,

2003). The residual material was instead stored in a dark, refrigerated room at c. 10 °C with ambient air humidity higher than 80%, to achieve devitalization.

2.2 Storage and post-storage assessment of lichen vitality

During storage and at the end of the storage, the photosynthetic activity of algal populations was occasionally assessed by chlorophyll fluorescence emission (Chl_aF) measurements on terminal lobes of randomly selected thalli, to assess their health status (Tretiach et al. 2007). Chl_aF was assessed in terms of the maximum quantum yield of primary photochemistry in dark adapted samples, using the parameter F_v/F_m as a proxy for the efficiency of photosystem II (Candotto Carniel et al. 2017).

Dark-stored thalli were let air drying at room temperature, whereas thalli stored at -20°C were thawed in silica for 24 hours. F_v/F_m values were assessed on 60 lobes per set, each one detached from a randomly chosen thallus, by selecting scarcely isidiate terminal lobes of 2.5 cm length. Prior to the Chl_aF measurements, lobes were hydrated in jars for 48 h at c. 100% relative humidity (RH), 18 °C, and 30 µmol photons m^{-2} s^{-1} for 14 h per day. Once hydrated, lobes were rinsed for 3 minutes in dH_2O, gently shaken to remove the excess water, then dark-adapted for 30 min.

Chl_aF measurements were carried out with a Photosynthetic Efficiency Analyzer Fluorimeter Handy-PEA (Hansatech, King's Lynn, UK). Lichens were considered either fully vital (henceforth, "living - *L* - samples") or dead (henceforth, "dead - *D* - samples") when F_v/F_m exceeded 0.5 or it was lower than 0.1 (Jensen 2002). As expected, the long-term storage at -20°C was effective in preserving the vitality of thalli; contextually, the protracted dark storage at high ambient air humidity led to a successful devitalization (Supplementary Fig. S1).

2.3 Study area and lichen transplant

After the vitality assessment, a sufficient amount of samples from the two bulk sets was selected to assess the elemental composition of living and devitalized lichen material prior the transplant study. These samples were not exposed in the study area ("unexposed" or "pre-exposure" samples), but refrozen at -20°C until retrieving transplanted *L* and *D* samples. The transplant area covers c. 40 km^2 in a typical mixed land use plain located at the foot of the Carnic Pre-Alps (NE Italy) (Kodnik et al. 2015). It includes a medium-extent urban centre (Maniago) and three smaller towns (Arba, Cavasso Nuovo and Fanna). The main potential anthropogenic pollution sources are a large industrial park, an isolated medium-sized cement plant (Supplementary Fig. S2), vehicular traffic, and agricultural activities (Kodnik et al. 2017). In the study area, the elemental and PAH deposition patterns were repeatedly assessed through native and transplanted lichens (Tretiach and Baruffo 2001a; Tretiach and Pittao 2008; Kodnik et al. 2015, 2017).

In this study, 40 transplant sites were selected according to the systematic sampling design originally adopted in Kodnik et al. (2015, 2017) (Supplementary Table S1, Supplementary Fig. S2). Thirty-seven sites were located at the knots of a 700 m step grid, and three further in the nearby towns of Arba, Cavasso and Maniago.

A week before the field exposure, *L* and *D* thalli were mounted on exposure devices. From three to six thalli were secured with plastic cable ties to wooden rods (120 cm long, 0.5 cm Ø) previously

subjected to dH_2O washing. Overall, 80 exposure devices were assembled, 40 bearing *L* thalli and 40 bearing *D* ones. Immediately after their preparation (June 13th, 2018), paired (*L-D*) exposure devices were placed at each transplant site, attached to the external branches of deciduous trees at c. 4 m above the ground, within 8 hours of field work. After 8 weeks (August 18th), all samples were retrieved, with the exception of the *D* sample exposed at site 7D, that was missing.

After their retrieving, the health status of samples was again assessed by Chl_aF measurement on 60 lobes per set, as described above (Sect. 2.2). After the exposure, *L* samples stayed vital, although F_v/F_m mean values lowered due to stressing field conditions (Supplementary Fig. S1), in accordance with previous observations on *P. furfuracea* transplanted in summertime, irrespective the pollutant loads (e.g., Tretiach et al. 2007; Pirintsos et al. 2011).

2.4 Sample processing and element content determination

After their retrieving, samples were transported to the laboratory and left to dry out at room temperature for 24 h. Afterwards, terminal lobes homogenous in size (15 - 25 mm) were selected and grinded for 4 minutes at 30Hz with a mixer mill Retsch MM 400. The resulting powder (c. 1 g per sample) was stored in pre-labelled polypropylene tubes and kept in silica until the analytical determination.

Element content determination was performed at Bureau Veritas Mineral (BVM) laboratories (Arkansas, USA). Grinded samples of *P. furfuracea* were subjected to a partial acid digestion with ACS-grade HNO_3 (1 hour), and Aqua Regia (ACS-grade HCl-HNO_3, volume ratio 1:3) in a boiling water bath (95°C, 1 hour). The concentrations of 24 elements (Al, As, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Mo, Na, Ni, P, Pb, S, Sb, Sr, Ti, Zn) were measured through Inductively Couple Plasma Mass Spectroscopy (ICP-MS), with a Perkin Elmer Elan 6000 ICP-MS. The resulting concentration values were expressed on a dry weight basis ($\mu g\ g^{-1}$ DW).

In order to assess the accuracy of analytical procedures, BVM laboratories analysed aliquots of two in-house reference materials (CDV-1 and V16, plant leaves), with the same protocol adopted for the experimental samples. Accuracy results were expressed in terms of mean recovery percentages (Supplementary Table S2).

2.5 Data analysis

Descriptive statistics were calculated for the concentrations of 24 target elements measured in unexposed and exposed *L* and *D* samples (Supplementary Table S3). Afterwards, element concentration values in exposed samples were expressed with respect to those of unexposed ones in terms of the so-called *Exposed-to-Unexposed* ratio (*EU* ratio; Cecconi et al. 2019a), and the same descriptive statistics were calculated for dimensionless *EU* ratios (Table 1).

Explorative multivariate statistics (PCA and hierarchical CA) were performed on the *EU* ratio data matrix of *L* and *D* samples. Firstly, a PCA was performed on the matrix 78 × 24 (39 *L* samples plus 39 *D* samples × 24 elements). The four quantitative levels of the factor '*land use*' (U, urban; I, industrial; R, rural; N, natural; Supplementary Methods S1.1) were also included in the analysis as supplementary variables and shown as vectors in the principal component (PC) space of elements. The two levels of the factor '*sample set'* (i.e., *L* and *D*), were inserted as binary dummy variables,

indicating the vitality of lichen samples. Dummy and supplementary variables were not used to calculate the principal components (PCs) but plotted on the ordination space based on their correlations with the PCs (Legendre and Legendre, 1998).

For comparative purposes, *EU* ratio data derived from living and dead samples were also organized in two *distinct* matrices 39 × 24 (39 samples × 24 elements for either *L* or *D* sets). The variables (elements) and cases (sites) of such matrices were subjected to hierarchical CAs using as distance measure and clustering algorithm, respectively, Pearson's 1 – r and the complete linkage, and the Euclidean distance and the Ward's method. Then, for the element groups and the site clusters, among-group/among-cluster significant differences were tested by non-parametric Kruskal-Wallis ANOVA and Dunn's post-hoc test.

In order to address the effect of the lichen vitality on the accumulation of single elements and to assess potential interpretational differences deriving by the use of living and dead samples, significant differences between median *EU* ratios of *L* and *D* samples were tested by Wilcoxon signed rank test (the same was done for the median concentrations of unexposed and exposed *L* and *D* sample sets; Supplementary Table S3). The lichen vitality was considered to have a systematic effect when the element-specific *EU* ratio was higher in either *L* or *D* in more than 80% of sites. Accordingly, mean *EU* ratios were used to classify the accumulation of the 24 target elements, either overall, or site by site, on the basis of the bioaccumulation scale available for 8-week transplant applications (Supplementary Table S4).

All data analyses and graphics were performed with the software packages QGIS 2.18.17 'Las Palmas', Statistica v. 10 (StatSoft Inc., Tulsa, OK, USA) and R (R Core Team 2013). Statistical significance was tested at $\alpha = 0.05$ in all cases.

3. Results

3.1 Multivariate assessments

The first and second principal components (PC 1, PC 2) of the multivariate space describe 35.8% and 13. 9% of variance (Fig. 1). PC 1 is negatively associated with the *EU* ratio of most elements (Al, Ca, Cd, Co, Fe, Hg, Pb, Sr, Ti and Zn) and positively with that of K, Na, P and S. Moreover, this axis is negatively and positively correlated with living and dead lichen samples (their projection on PC 1 being ± 0.72), therefore indicating a higher bioaccumulation of elements placed at negative scores of PC 1 in dead samples, and contextual higher *EU* ratios of K, Na, P and S in living samples. PC 2 is instead positively correlated with Bi, Cr, Mo, Ni and Sb (with negative PC 1 scores), as well as with K, Na, S and P (with positive PC 1 scores) (Fig. 1a). Concerning landcover categories in the surroundings of the transplant sites, the industrial land use is respectively negatively/positively correlated with PC 1/PC 2, suggesting an enhanced accumulation (higher *EU* ratios) of Al, Bi, Ca, Cd, Co, Cr, Fe, Hg, Mo, Ni, Pb, Sb, Sr, Ti, and Zn. Natural land use is consistently positively/negatively correlated with PC 1/PC 2, suggesting the lowest loss (higher *EU* ratios) of physiological elements (mostly K and P) as well as the lowest accumulation of Bi, Cr, Mo, Ni and Sb.

Lichen samples segregate according to their set, with *L* samples mostly placed in the first quadrant, and *D* samples mostly occupying the third quadrant (Fig. 1b).

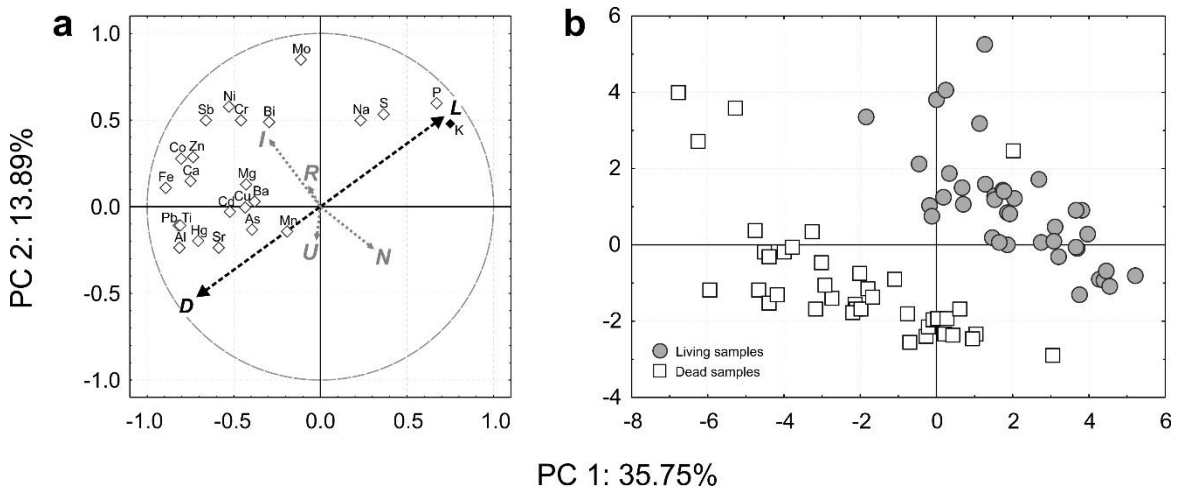


Figure 1. Principal Component Analysis (PCA) based on *EU* ratio data of elements (left) and Pseudevernia furfuracea samples (right). In the PC space of elements, sample sets and land use categories are represented as supplementary variables (black dotted arrows: L - living samples, D - dead samples; grey dotted arrows: I - industrial, U - urban, R - rural, N - natural landcover; Supplementary Methods S1.1, Table S1).

The Cluster Analysis (CA) of elements performed on the *EU* ratios of *L* and *D* sets produced dendrograms with comparable topologies (Fig. 2a). At the same linkage distance, four groups can be identified in both cases, with elements co-occurring within each group of the two dendrograms. Therefore, matching groups were labelled with the same roman numeral and a superscript reflecting the sample set (I^L-I^D, ... IV^L-IV^D). In particular, Al, Fe and Ti (lithogenic elements) plus Cd and Hg, Bi, Cr, Mo and Ni (heavy metals associated to steel work industry), Ba, Ca and Mg (alkaline earth metals) plus Cu and Pb, as well as K and P (physiology-related elements), are shared within group I, II, III, and IV, respectively.

The results of the non-parametric Kruskall-Wallis ANOVA reveal that the *EU* ratios of element groups significantly differ among the sample sets (see bar charts at the bottom of Fig. 2a). Lithogenic elements of group I show the largest significant differences between *EU* ratios of *L* and *D* samples, with *D* samples characterized by the highest values, so as group III, although with more limited inter-set differences. Differently, group II shows significantly higher *EU* ratios in *L* samples. Physiological elements of group IV are instead not accumulated ("Absence of bioaccumulation") by both sample sets, although their loss is substantially higher in *D* samples (Fig. 2a). Overall, averaged *EU* ratios for different groups of elements in both sample sets never exceed the upper threshold of "low bioaccumulation" class (*EU* ratio $\leq$ 1.9; class 2 of the Bioaccumulation Scale; Fig. 2a; Supplementary Table S4), therefore highlighting generally low elemental depositions over the study area.

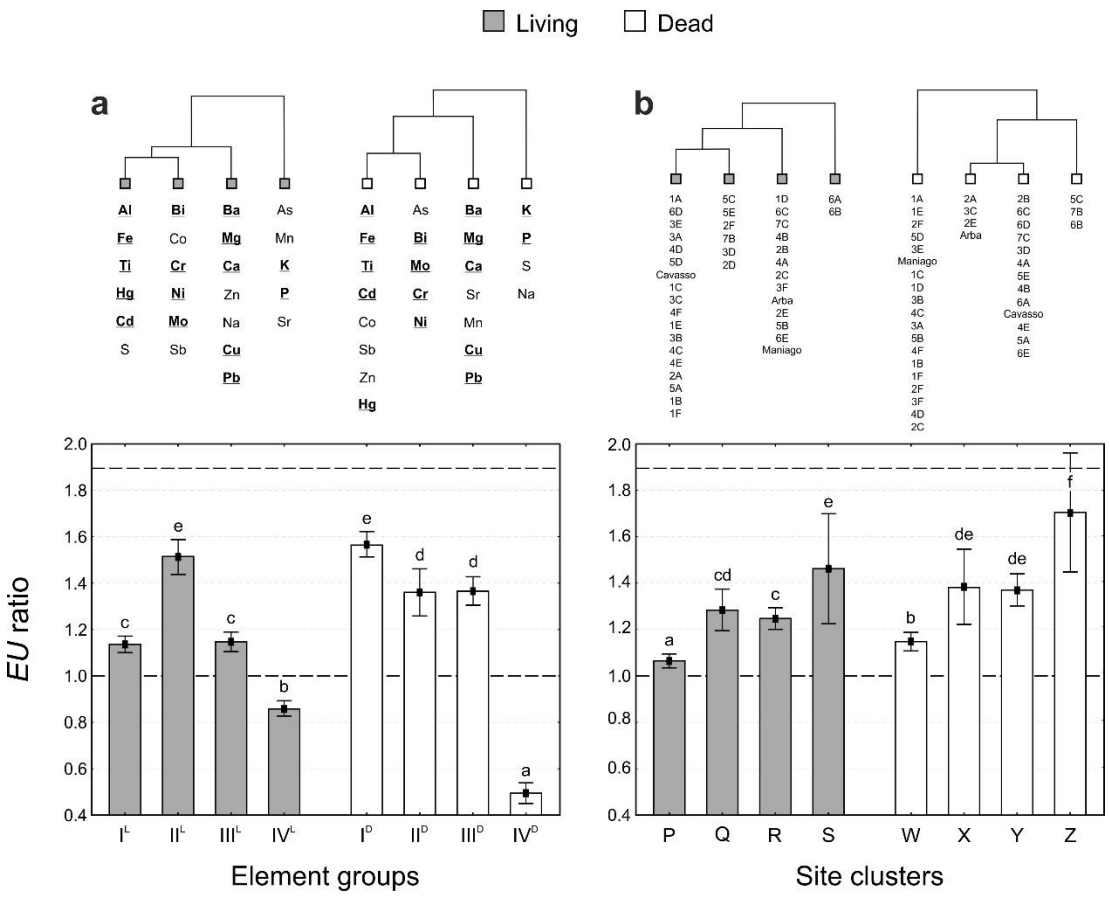


Figure 2. Cluster Analysis (CA) of elements (a) and sites (b) with bar charts showing mean *EU* ratio values for different element groups (a) and site clusters (b) (error bars indicate 95% confidence intervals). Black dotted lines show the *EU* thresholds of bioaccumulation classes for 8 week-transplants (Supplementary Table S4). Letters above bars indicate among-group/cluster significant differences (Kruskal-Wallis ANOVA and Dunn's post hoc test). Elements shared by matching groups are underlined and reported in bold.

By cutting the site dendrograms at the same linkage distance, four clusters of comparable dimensions are still formed for either *L* or *D* samples (Fig. 2b). However, in this case, the two dendrograms do not share the same overall topology, therefore these were labelled with different letters. Although site clusters are comparable in size (P, Q, R and S include 18, 6, 13 and 2 sites respectively, whereas W, X, Y and Z include 19, 4, 13 and 3 sites), a weaker match can be noticed between the cluster composition of *L* and *D* sets (Fig. 2b). In the three clusters sharing sites, mean *EU* ratio values differ among the sample sets with *D* samples exhibiting significantly higher values. Averaged *EU* ratios for different site clusters never exceed the "low bioaccumulation" class (*EU* ratio $\leq$ 1.9; class 2 of the Bioaccumulation Scale; Fig. 2b; Supplementary Table S4). In particular, although significantly differing, clusters P and W (sharing the highest number of samples) show the lowest mean *EU* levels. Clusters S and Z (sharing the industrial site 6B) show instead the highest bioaccumulation: in this case, and limited to cluster Z (*D* samples), the upper 95% confidence limit falls in the "moderate bioaccumulation" class (1.9 < *EU* ratio $\leq$ 2.7; class 3 of the Bioaccumulation Scale; Fig. 2b; Supplementary Table S4). An intermediate situation can be highlighted for clusters Q and R of *L* samples and for clusters X and Y of *D* samples, respectively. Indeed, within the same sample set the averaged *EU* ratio values of these clusters do not differ (Fig. 2b).

3.2 Living *vs* dead: single-element accumulation performance and transplant site alteration

When addressing single elements, Al, As, Ca, Cd, Co, Cu, Fe, Hg, K, Mg, Mo, Na, P, Pb, S, Sr, Ti, Zn show significant *EU* ratio differences between sample sets (Table 1; Fig. 3).

Table 1. Descriptive statistics (mean ± standard deviation, 95% confidence interval, median and range) of element *EU* ratios in living (*L*) and dead (*D*) samples, with the output of the Wilcoxon test for paired samples on *EU* ratio data (*p*-values < 0.05 are reported in italic).

Element	Living samples (*L*)				Dead samples (*D*)				Wilcoxon	
	Mean ± SD	C.I. 95%	Median	Range	Mean ± SD	C.I. 95%	Median	Range	Z	*p*-value
Al^{+}	1.16 ± 0.24	1.08 - 1.24	0.95	0.95 - 1.43	1.73 ± 0.3	1.63 - 1.83	1.88	1.25 - 2.50	5.30	1.1×10^{-7}
As	0.65 ± 0.22	0.57 - 0.72	0.53	0.53 - 1.05	0.99 ± 0.59	0.80 - 1.18	0.91	0.45 - 2.27	2.39	*0.017*
Ba	1.11 ± 0.21	1.05 - 1.18	1.09	0.79 - 1.61	1.19 ± 0.20	1.12 - 1.25	1.22	0.82 - 1.55	1.77	0.076
Bi	1.67 ± 0.77	1.42 - 1.92	1.50	1.00 - 3.50	1.58 ± 0.72	1.34 - 1.81	1.50	1.00 – 4.00	1.09	0.276
Ca^{+}	1.05 ± 0.19	0.99 - 1.11	1.03	0.74 - 1.58	1.24 ± 0.28	1.15 - 1.33	1.20	0.81 - 1.83	3.88	1.0×10^{-4}
Cd	1.18 ± 0.33	1.07 - 1.29	1.09	0.76 - 2.83	1.35 ± 0.21	1.28 - 1.42	1.31	0.83 - 1.79	3.19	*0.001*
Co	1.20 ± 0.20	1.14 - 1.27	1.22	0.76 - 1.68	1.39 ± 0.23	1.32 - 1.47	1.42	0.79 - 1.81	3.88	1.0×10^{-4}
Cr	1.22 ± 0.27	1.13 - 1.30	1.19	0.77 - 2.04	1.35 ± 0.51	1.18 - 1.51	1.21	1.03 - 3.53	1.27	0.204
Cu	1.67 ± 0.48	1.52 - 1.83	1.57	1.12 - 3.34	2.08 ± 0.85	1.80 - 2.35	1.89	1.26 - 5.83	3.06	*0.002*
Fe^{+}	1.32 ± 0.25	1.24 - 1.41	1.33	0.90 - 1.72	1.71 ± 0.30	1.61 - 1.81	1.70	0.98 - 2.47	4.90	9.7×10^{-7}
Hg^{+}	0.89 ± 0.12	0.85 - 0.92	0.88	0.69 - 1.12	1.16 ± 0.20	1.09 - 1.22	1.11	0.85 - 1.60	4.94	7.8×10^{-7}
K°	0.76 ± 0.13	0.72 - 0.80	0.76	0.51 - 1.01	0.24 ± 0.11	0.20 - 0.27	0.22	0.07 - 0.66	5.44	5.3×10^{-8}
Mg	1.16 ± 0.16	1.11 - 1.22	1.14	0.85 - 1.54	1.24 ± 0.20	1.18 - 1.31	1.22	0.91 - 1.82	2.22	*0.026*
Mn	1.01 ± 0.26	0.91 - 1.11	0.99	0.48 - 1.57	1.10 ± 0.37	0.98 - 1.22	1.00	0.56 - 2.72	1.16	0.247
Mo°	1.54 ± 0.53	1.37 - 1.71	1.46	0.80 - 3.87	1.17 ± 0.56	0.99 - 1.36	0.95	0.71 - 3.89	4.35	1.3×10^{-5}
Na°	0.76 ± 0.25	0.67 - 0.84	1.00	0.50 – 1.00	0.55 ± 0.19	0.49 - 0.61	0.45	0.45 - 0.91	4.48	7.5×10^{-6}
Ni	1.46 ± 0.52	1.29 - 1.63	1.28	0.90 - 3.33	1.72 ± 0.93	1.42 - 2.02	1.47	0.88 - 5.44	1.90	0.058
P°	0.98 ± 0.16	0.93 - 1.04	0.97	0.60 - 1.36	0.33 ± 0.17	0.28 - 0.39	0.31	0.22 - 1.30	5.43	5.7×10^{-8}
Pb^{+}	1.12 ± 0.17	1.06 - 1.17	1.07	0.81 - 1.59	1.48 ± 0.23	1.41 - 1.56	1.46	0.91 - 2.08	5.19	2.1×10^{-7}
S°	1.03 ± 0.18	0.97 - 1.08	1.01	0.63 - 1.39	0.85 ± 0.14	0.81 - 0.90	0.81	0.81 - 1.61	4.05	5.2×10^{-5}
Sb	2.00 ± 0.62	1.80 - 2.20	2.00	0.80 - 3.60	2.26 ± 0.86	1.98 - 2.54	2.38	0.95 - 5.24	1.91	0.056
Sr	0.91 ± 0.20	0.85 - 0.97	0.89	0.59 - 1.62	1.25 ± 0.41	1.11 - 1.38	1.13	0.70 - 2.46	4.17	3.0×10^{-5}
Ti^{+}	1.24 ± 0.25	1.16 - 1.32	1.22	0.98 - 1.95	1.91 ± 0.30	1.74 - 1.99	2.00	1.00 - 2.33	4.76	1.9×10^{-6}
Zn	1.16 ± 0.15	1.12 - 1.21	1.16	0.93 - 1.51	1.31 ± 0.20	1.24 - 1.37	1.26	1.01 - 1.98	3.52	4.4×10^{-4}

° Elements showing higher *EU* ratio values in *L* samples in more than 80% of transplant sites (Supplementary Fig. S4).
⁺ Elements showing higher *EU* ratio values in *D* samples in more than 80% of transplant sites (Supplementary Fig. S4).

As already highlighted at the cluster level, physiological elements (K, Na, P, S) were generally characterized by "absence of bioaccumulation", but significantly higher elemental losses occurred in *D* samples during the exposure. *L* samples had significant higher *EU* ratios limited to Mo, which, by itself, determined the significant higher median *EU* ratio of group II^{L} (Sect. 3.1; Fig. 2a). Concerning Mg, both sample sets were characterized by "low bioaccumulation", but *D* samples showed slight, although significant, higher *EU* ratios (Fig. 3). Overall, *D* samples were more effective in accumulating lithogenic elements (Al, Ca, Fe, Ti) and As, Cd, Co, Cu, Hg, Pb, Sr and Zn.

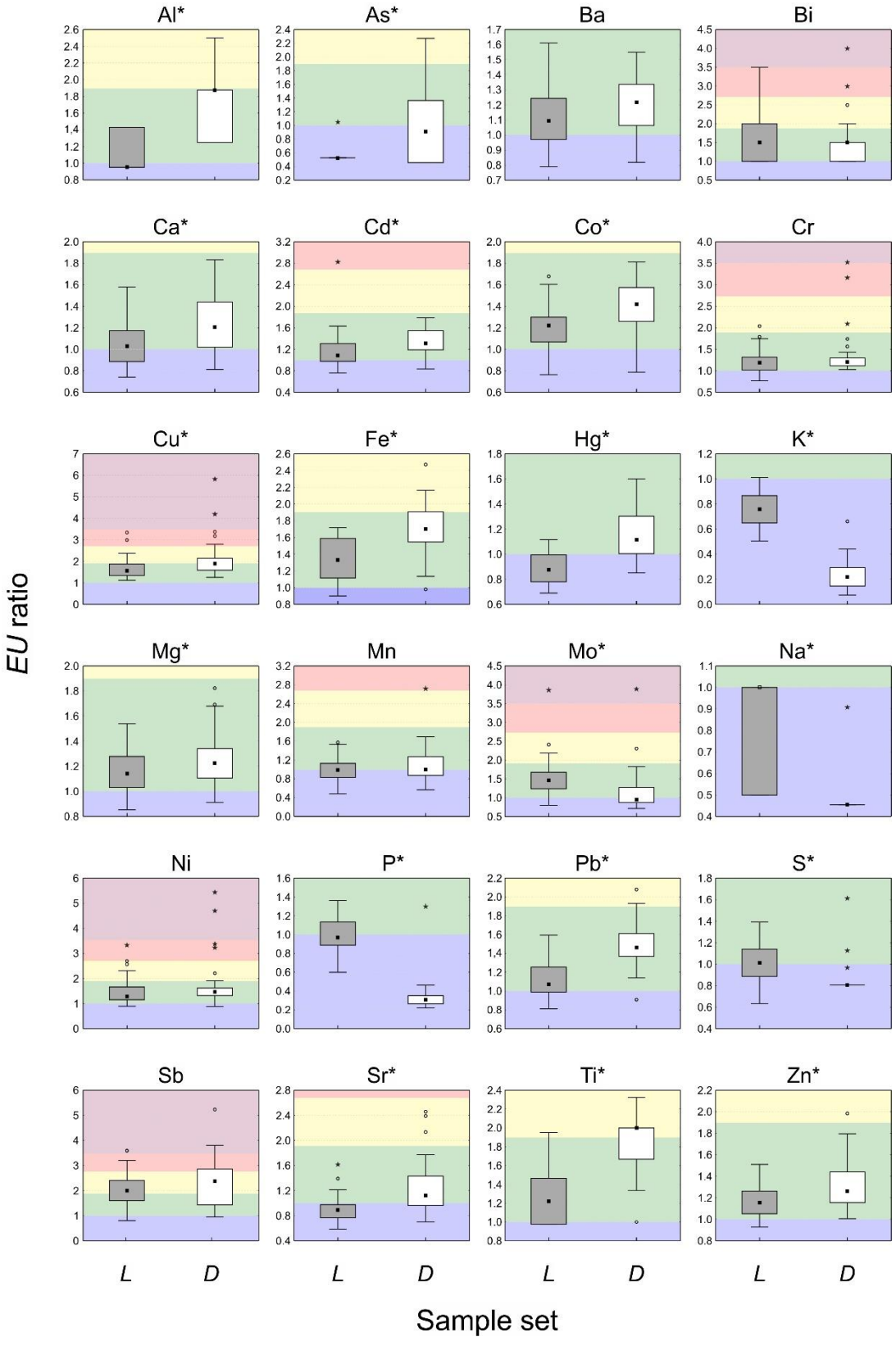


Figure 3. Boxplots of *EU* ratio data of living (*L*, grey) and dead (*D*, white) *P. furfuracea* samples for 24 target elements. Data refer to median, first and third quartiles, and non-outlier ranges (outliers and extreme values are highlighted by circles and stars, respectively). Asterisks next to the element name indicate significant differences between the sample sets (Wilcoxon test; Table 1). Background is coloured according to the *EU* range of bioaccumulation classes (Supplementary Table S4).

A consistent effect of lichen vitality was highlighted over the study area for a subset of elements exhibiting between-set significant differences. Indeed, *EU* ratios of K, Mo, Na, P and S were higher

in *L* samples in more than 80% of transplant sites, whereas the opposite was found for Al, Ca, Fe, Hg, Pb and Ti (Supplementary Fig. S3).

When the interpretative scale (Supplementary Table S4) was used to classify the mean *EU* ratios of element content in *L* and *D* samples, this led to different class attribution for some elements (Supplementary Fig. S4). Indeed, the mean *EU* ratio of S in *L* and *D* samples was attributed to "low bioaccumulation" and to "absence of bioaccumulation class", respectively. The opposite was instead observed for Cu, Hg, Sr, and Ti. The mean *EU* ratio values calculated for Hg and Sr in *L* samples showed "absence of bioaccumulation" (*EU* ratio ≤ 1; class 1) and "low bioaccumulation" (*EU* ratio ≤ 1.9) in *D* ones, whereas those of Cu and Ti were characterized by "low bioaccumulation" in *L* samples and by "moderate bioaccumulation" (*EU* ratio ≤ 2.7; class 3) for *L* and *D* samples, respectively (Supplementary Fig. S4). With the exception of Cu, these elements exhibited higher *EU* ratios in more than 80% of transplant sites, in either *L* (for S) or *D* (for Hg, Sr and Ti) sample sets.

When *EU* ratios of single elements were addressed site per site, the results obtained by different sample sets depicted a general pattern of low pollutant depositions, irrespective the use of *L* and *D* samples. The majority of transplant sites were accordingly characterized by the predominance of "low" or "absence of" bioaccumulation. Indeed, when considering *L* samples, only two sites out of 39 were characterized by less than 80% of classes 1 and 2 (6A and 4B), whereas 11 sites were exclusively characterized by such classes (Supplementary Fig. S4). When referring to *D* samples, eight sites out of 39 were characterized by less than 80% of classes 1 and 2, whereas six sites were exclusively characterized by such classes (Supplementary Fig. S4).

However, when focusing on the situations of alteration the use of different sample sets also determines some major differences. Indeed, with *L* samples only eight sites out of 39 were characterized by more than 10% of classes 3, 4 and 5 (from "moderate" to "extreme" bioaccumulation; Supplementary Fig. S4). Instead, when referring to *D* samples, more than a half of transplant sites were so characterized, with 19 sites showing such pattern (Supplementary Fig. S4).

4. Discussion

4.1 Bioaccumulation capacity of living and dead samples

After the exposure in the study area, living and dead *Pseudevernia furfuracea* samples showed different elemental content. Indeed, the statistical analysis of *EU* data characterizing the experimental sets highlighted a higher enrichment of Al, Ca, Fe, Ti, As, Cd, Co, Cu, Hg, Pb, Sr, Zn and a higher loss of K, Na, S, and P by dead thalli, whereas living samples were more effective only in accumulating Mo. This element is an essential micronutrient for almost all biological systems (especially bacteria, but also eukaryotes), which holds key positions in several enzymes involved in carbon, nitrogen and sulphur metabolism (Peng et al. 2018). However, the role of Mo as enzymatic cofactor, by itself, does not explain an enhanced accumulation by healthy *P. furfuracea* thalli, although suggesting the possibility for interesting *in vitro* research to clarify the accumulation behaviour of Mo in lichen ecosystems.

The higher loss of K, Na, S, and P by dead samples is in accordance with previous observations of impairment of lichen intracellular uptake mechanisms caused by ultrastructural/physiological

damage (e.g., Tretiach et al. 2007; Spagnuolo et al. 2011; Corapi et al. 2014). Indeed, when plasma membranes are severely damaged, the cytoplasmatic immobilization of ions by intracellular binding matrix may result impaired (Tyler 1989), causing the loss of ions (Asta and Garrec 1980; Bargagli and Mikhailova 2002).

In lichens, a large proportion of airborne trace elements is mainly accumulated by the extracellular entrapment of particulate matter (Tretiach et al. 2011) occurring within the loose hyphal weft of the medulla, which also prevents toxicity at cell level (Bargagli 1998). Therefore, the relative importance of the particle entrapment in dead matrices may result substantially enhanced due to the empty cell volumes, leading to an increased availability of ion binding sites at cell wall level (Richardson et al. 1985). Indeed, lichen cell walls contain a plurality of compounds (e.g., chitin, glucans, oxalates, polyketides) with several poly-anionic functional groups able to bind metal ions (Sarret et al., 1998). There are several evidences that elements with high affinity for these functional groups, especially Al, Cu, Hg, Fe, Pb, and Ti (Nieboer et al. 1978; Bargagli and Mikhailova 2002) may continue to be accumulated in dead thalli (Chettri et al. 1997).

Our findings were in general agreement with the results achieved for other lichen species under different experimental conditions. For instance, Nieboer et al. (1976) investigated the metal uptake by *Umbilicaria muhlenbergii* (Ach.) Tuck. *in vitro*, proving that the uptake of Ni from solutions of $NiCl_2$ was merely physicochemical. Indeed, dead thalli accumulated the metal to a slightly greater extent (Nieboer et al. 1976). In our transplant experiment, after the 8-week exposure, dead *P. furfuracea* samples had higher mean *EU* ratio for Ni, although not significantly (Table 1). Moreover, the metal accumulation was higher in dead samples in 25 out of 39 sites (64% of cases), also producing from single- to three-step differences in bioaccumulation classes at sites 3A, 6B, 4E, 5D, 7B and 5C (i.e., *L* vs *D* samples: classes 2-1, 4-3, 4-2, 3-1, 5-3, respectively).

Chettri et al. (1997) investigated the uptake of Cu, Pb and Zn by *Cladonia convoluta* (Lam.) and *C. rangiformis* (Hoffm.) from solutions of $Pb(NO_3)_2$, $CuCl_2$ and $ZnCl_2$. The uptake of Cu and Pb was higher in dead *Cladonia* thalli, whereas the opposite was found for Zn, whose content is usually higher in the intracellular fraction of living thalli (Fortuna et al. 2017). Chettri et al. (1997) also highlighted that Zn suffers competitive uptake, being affected by the co-occurrence of Cu and Pb in the medium. These results match our findings for Cu and Pb; limited to Zn, we highlighted an overall higher accumulation by dead thalli (also revealed by the other single work targeting such issue in *P. furfuracea*; see *infra*). The fully controlled experimental conditions of Chettri et al. (1997), along with the frequently proven species-specificity of elemental accumulation (Nimis et al. 2001; Tretiach and Baruffo 2001b; Minganti et al. 2003; Bergamaschi et al. 2007), may easily explain the discrepancy.

The accumulation efficiency of living and dead *P. furfuracea* was also investigated by Adamo et al. (2007) in a 6-week transplant experiment at two urban sites. The authors demonstrated that the accumulative efficiency of living samples was not higher than that of dead ones, showing the major role of atmospheric particulate, irrespective of organism vitality. For both exposure sites they reported slightly higher bioaccumulation levels in devitalized samples for Al, Ca, Cd, Cr, Cu, Mn, and Zn, with the exception of Hg, instead showing higher levels in living thalli. Overall, our results match previous findings: indeed, all elements showed higher bioaccumulation in dead samples, either significantly (Al, Ca, Cd, Cu and Zn) or not (Cr and Mn, Table 1). The differences observed

for Hg may be explained by the interplay of peculiar behaviour of this element in the atmosphere and the different pollutant loads affecting the exposure sites. It is feasible that the low levels observed in this work and by Adamo et al. (2007) may derive from different relative contributions of Hg forms (i.e., gaseous or associated to particulate; Bargagli 2016; Keeler et al. 1995). If so, gaseous Hg would be mostly actively accumulated at intracellular level, resulting in an enhanced bioaccumulation by living thalli (as in Adamo et al. 2007), whereas the accumulation of Hg adsorbed on airborne particulate matter would be enhanced in dead matrices by physical entrapment (as possibly in this study).

4.2 Pollutant loads and lichen health: the risk of interpretative bias

Overall, the study area was not exposed to high and uniform pollutant loads. Indeed, when classified according to the new interpretative scale for lichen transplants, the majority of *EU* ratio values of both living and dead samples were associated to "low" or "absence of" bioaccumulation classes (*EU* ratio ≤ 1.9). Only Cu and Ti in dead samples, as well as Sb in both living and dead ones exceeded class 2 (Supplementary Table S4 and Fig. S3). Therefore, besides the cautious terminology of the interpretative scale (focusing on lichens - i.e., "bioaccumulation levels" - rather than on "environmental alteration"; Cecconi et al. 2019a), the historically acknowledged link between lichen elemental enrichment and air pollution (e.g., Herzig et al. 1989; Sloof 1995; Van Dobben et al. 2001) expressly indicates the absence of a homogeneous alteration in the study area. This is especially true for As, Hg, and Pb, elements whose atmospheric concentration is targeted by the European Air Quality Directives (2008/50/EC4, 2004/107/EC5). Despite such general pattern, a small set of elements - Bi, Cu, Ni, and Sb - was characterized by substantial bioaccumulation (*EU* ratio > 2.7; class 3) at several sites in either living or dead lichens. Limited to Sb, it must be acknowledged that its recovery is far from being satisfactory (47.6%; Supplementary Table S2), indicating a substantial underestimation of lichen enrichment (and thus of Sb pollution) in the study area. Instead, Cd, Cr and Ti showed such levels limited to single sites in living samples (Cd: class 4 at Maniago; Cr: class 3 at 6A; Ti: class 3 at 2D), or to a higher number of sites in dead samples (Cr: class 3 at 4E, class 4 at 5C and class 5 at 7B; Ti class 3 at 20 sites; Supplementary Fig. S4). All such elements are generally considered tracers of coal combustion (Van de Velde et al. 1999), also related to iron, steel, and ferro-alloy industrial processing (Tretiach and Pittao 2008; Brunialti and Frati 2014). Consistently, Cr, Mo, Ni and Sb showed the highest bioaccumulation levels within or near the industrial park, along with the highest concentrations of Fe, Pb, and Zn, however characterized by negligible depositions over the whole territory.

Despite an overall accordance of results obtained by using different sample sets, interpretative differences arise in terms of depositional patterns (which is substantiated by different structures of the two site dendrograms; Fig. 2b) and severity of metal-rich particulate pollution (signals are enhanced by using devitalized samples). In this respect, and concerning Fe, Hg and Pb, it is worth to notice that these were the only elements whose pre-exposure concentration values significantly differ between the experimental sample sets (Supplementary Table S3). In particular, for Fe and Hg, the concentration values in unexposed *L* samples (U_L) were significantly higher than those of *D* samples ($U_L > U_D$), whereas the concentrations measured in exposed (E) *L* and *D* samples were fully comparable ($E_L \approx E_D$). In the case of Pb, besides higher pre-exposure concentration values in *L*

samples, also the concentrations of exposed samples differ, exhibiting the opposite pattern ($U_L > U_D$ and $E_L < E_D$). Strictly speaking, in case of limited elemental depositions, as in this case, significant differences observed in *EU* ratio denominators (*U* values) would not allow proper ratios comparison. Indeed, significantly higher denominators in *L* samples could, by themselves, produce lower *EU* ratios for such samples, possibly determining unreliable outcomes of statistical testing for such elements. Nonetheless, it must also be considered that, in such cases, *EU* ratios were higher in *D* samples in more than 80% of transplant sites (Table 1; Supplementary Fig. S3), and that *EU* ratios of *L* and *D* samples determined different bioaccumulation classes for 33%, 64% and 28% of sites (Supplementary Fig. S4), an indication that the consistent effect of the factor "lichen vitality" likely overcomes that of different pre-exposure concentrations values.

Another aspect that has to be taken into account is that metabolically active thalli may experience different degrees of physiological impairment during the exposure (Tretiach et al. 2007), depending on environmental and meteoclimatic conditions characterizing the study area and the initial health status of lichens (Piccotto et al. 2010, 2011; see the variation of F_v/F_m distributions in living samples before and after exposure; Supplementary Fig. S1). Although the bulk material is generally collected in a limited-extended, environmentally homogeneous background area, the intrinsic biological variability could still enlarge the "noise" associated to the bioaccumulation of transplanted samples. Indeed, the use of samples characterized by different physiological conditions could produce different results in terms of bioaccumulation classes, thus introducing an additional source of variability in the delicate stage of result interpretation. Since it is rarely feasible to regularly monitor the lichen vitality during the exposure in potentially large areas, lichen devitalization prior to the exposure would maximize the "signal-to-noise" ratio by contemporarily enhancing the element accumulation ("signal") and reducing result variability ascribable to variations in sample health ("noise").

Since established practices may not always be the best ones, especially when not meeting the needs of standardization, the possibility of sample devitalization deserves to be taken into consideration in the framework of active lichen biomonitoring. This is all the more true, since it was recently proved that devitalized *P. furfuracea* samples better accumulate PAHs compared to living co-specific samples and dead *Hypnum cupressiforme* ones, better allowing the detection of such frequently targeted pollutants at low concentrations (Capozzi et al. 2020).

5. Conclusions

This study demonstrated an enhanced performance of dead lichen matrices in accumulating particulate-related elements. For the first time such behaviour was proved in a large sample-sized transplant application, thus providing further evidence that passive uptake mechanisms play a major role in trace element bioaccumulation when the exposure time is limited to 2 months. In particular, higher accumulation capacity was revealed in dead lichens for Al, Ca, Fe, Ti, As, Cd, Co, Cu, Hg, Pb, Sr, Zn. Amongst these elements, a systematic effect of the lichen vitality was revealed for Al, Ca, Fe, Hg, Pb and Ti. Although both sample sets consistently depicted a non-alarming situation of pollutant depositions in the study area, their contextual use also highlighted some non-negligible discrepancies. Indeed, devitalized samples, although not subjected to traditional devitalization

treatments, are still capable of maximizing the accumulation signal, allowing to highlight the finest depositional patterns. Sanely, this capability has long been considered a desirable feature for moss matrices, which begs the question: why not to prefer dead lichens too?

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS S1

S1.1 Land use characterization of the transplant sites

The 40 transplant sites were characterized in terms of land use using the Corine Land Cover map 2012 (Bossard et al. 2000) in GIS environment. The percent cover of urban (2nd order level '11'), industrial (2nd order level '12'), rural (1st order level '2') and natural (2nd order level '31') landcover was estimated within buffers of 125 m radius centred on each site. Sites were accordingly classified as urban, industrial, rural or natural when the percent cover of the corresponding class was higher than 50% (Supplementary Table S1).

SUPPLEMENTARY TABLES S1-S4

Supplementary Table S1. List of transplant sites with alphanumeric identification codes (site ID), UTM coordinates, altitude and land use classification obtained considering the percent coverage of different CLC categories in circular buffers of 125 m radius centred in the transplant sites (Supplementary Methods S1.1).

Site ID	UTM coordinates		Altitude (m a.s.l.)	Land use
	E	N		
1A	2343347	5116507	279	Natural
1B	2344025	5116735	324	Natural
1C	2344633	5116969	382	Natural
1D	2345383	5116934	299	Natural
1E	2346008	5117171	279	Urban
1F	2346755	5117276	270	Urban
2A	2343465	5115852	276	Urban
2B	2344060	5116095	270	Rural
2C	2344853	5116292	264	Rural
2D	2345549	5116006	253	Rural
2E	2346153	5116443	257	Rural
2F	2346845	5116601	254	Rural
3A	2343651	5115202	276	Urban
3B	2344315	5115216	267	Rural
3C	2344981	5115604	259	Rural
3D	2345739	5115575	248	Rural
3E	2346314	5115862	241	Rural
3F	2347092	5115850	242	Rural
4A	2343794	5114313	275	Urban
4B	2344503	5114672	263	Rural
4C	2345117	5114753	253	Rural
4D	2345854	5114939	236	Rural
4E	2346543	5115093	231	Rural
4F	2347088	5115190	229	Rural
5A	2343667	5113708	275	Rural
5B	2344595	5114199	264	Rural
5C	2345243	5114040	253	Rural

Supplementary Table S1 (continued)

Site ID	UTM coordinates		Altitude (m a.s.l.)	Land use
	E	N		
5D	2346052	5114223	240	Rural
5E	2346677	5114436	228	Rural
6A	2343855	5112999	268	Industrial
6B	2344703	5113241	257	Industrial
6C	2345404	5113217	248	Rural
6D	2346009	5113620	241	Rural
6E	2346775	5113701	231	Rural
7B	2344650	5112723	258	Rural
7C	2345533	5112752	245	Urban
7D	2346373	5112839	232	Rural
Arba	2349472	5112557	210	Urban
Cavasso Nuovo	2348230	5118263	278	Urban
Maniago	2341823	5114748	303	Urban

Supplementary Table S2. Lower Limit of Detection (LoD; μg g^{-1}) and recovery percentages (calculated as the percentage ratio between the measured and the expected values) for two in-house standard materials (i.e., V16 and CDV-1). Recovery percentages lower than 70% are highlighted in bold.

Element	LoD	Standard materials	
		V16	CDV-1
Al	100	95.4	86.7
As	0.1	89.6	82.1
Ba	0.1	92.4	98.2
Bi	0.02	-	-
Ca	100	96.7	93.3
Cd	0.01	89.2	83.3
Co	0.01	76.6	86.3
Cr	0.1	72.6	91.5
Cu	0.01	80.1	84.5
Fe	10	81.0	95.8
Hg	0.001	100. 7	108.9
K	100	90.9	87.0
Mg	10	94.0	96.4
Mn	1	90.7	94.5
Mo	0.01	75.4	85.0
Na	10	**66. 7**	96.2
Ni	0.1	77.1	83.3
P	10	97.1	73.6
Pb	0.01	93.1	86.0
S	500	-	83.3
Sb	0.02	**47.6**	-
Sr	0.5	95.8	96.5
Ti	1	82.6	74.4
Zn	0.1	85.3	86.3

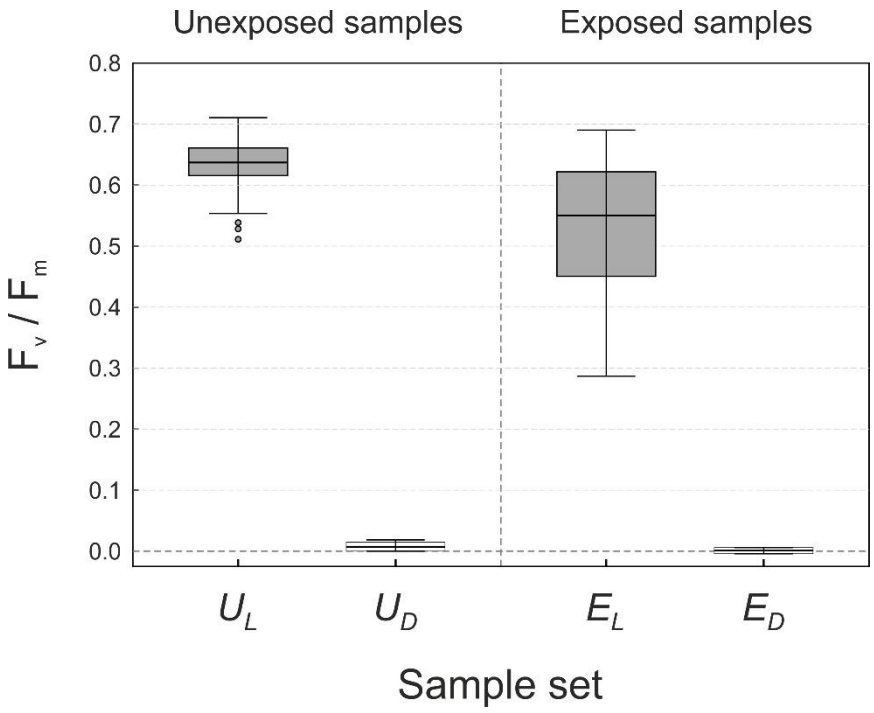
Supplementary Table S3. Descriptive statistics (mean ± standard deviation, 95% confidence interval, median and range) of element concentration data (μg g^{-1}) in living and devitalized *Pseudevernia furfuracea* samples, along with the output of the Wilcoxon test for paired samples (*p*-values < 0.05 are reported in italic). Element content values for unexposed (n = 10) and exposed samples (n = 39) are reported in the first and second row, respectively.

Element	Living samples				Devitalized samples				Wilcoxon	
	Mean ± SD	**C.I. 95%**	**Median**	**Range**	**Mean ± SD**	**C.I. 95%**	**Median**	**Range**	**Z**	***p*-value**
Al	210 ± 32	187 – 233	200	200 - 300	160 ± 52	123 - 197	200	100 - 200	1.83	0.068
	244 ± 50	227 - 260	200	200 - 300	277 ± 48	261 - 293	300	200 - 400	2.49	*0.013*
As	0.19 ± 0.07	0.14 - 0.24	0.20	0.10 - 0.3	0.22 ± 0.16	0.10 - 0.34	0.15	0.10 - 0.60	0.07	0.944
	0.12 ± 0.04	0.11 - 0.14	0.10	0.10 - 0.20	0.22 ± 0.13	0.18 - 0.26	0.20	0.10 - 0.50	3.39	*0.001*
Ba	12.8 ± 2.2	11.2 - 14.3	12.8	9.9 - 16.4	13.6 ± 2.7	11.7 - 15.4	12.9	10.3 - 19.4	0.87	0.386
	14.3 ± 2.7	13.4 - 15.1	14.0	10.1 - 20.6	16.1 ± 2.7	15.2 - 16.9	16.5	11.1 – 21.0	3.21	*0.001*
Bi	0.02 ± 0.00	-	0.02	0.02 - 0.02	0.02 ± 0.00	-	0.02	0.02 - 0.02	0.00	-
	0.03 ± 0.02	-	0.03	0.02 - 0.07	0.03 ± 0.01	-	0.03	0.02 - 0.08	1.17	0.242
Ca	4180 ± 922	3520 - 4840	4300	2700 - 5500	3820 ± 935	3151 - 4489	3500	2600 - 5800	0.82	0.415
	4377 ± 799	4118 - 4636	4300	3100 - 6600	4738 ± 1078	4389 - 5088	4600	3100 - 7000	2.14	*0.032*
Cd	0.09 ± 0.01	0.08 - 0.10	0.09	0.07 - 0.11	0.08 ± 0.01	0.08 - 0.09	0.09	0.06 - 0.10	1.48	0.138
	0.11 ± 0.03	0.10 - 0.12	0.10	0.07 - 0.26	0.11 ± 0.02	0.11 - 0.12	0.11	0.07 - 0.15	1.47	0.142
Co	0.13 ± 0.03	0.11 - 0.15	0.14	0.08 - 0.16	0.13 ± 0.02	0.11 - 0.14	0.13	0.09 - 0.16	0.36	0.721
	0.16 ± 0.03	0.15 - 0.17	0.16	0.10 - 0.22	0.18 ± 0.03	0.17 - 0.19	0.18	0.10 - 0.23	3.31	*0.001*
Cr	2.4 ± 0.3	2.1 - 2.6	2.3	2.0- 3.0	2.2 ± 0.3	2.0 - 2.4	2.2	1.9 - 2.7	0.51	0.612
	2.9 ± 0.6	2.7 - 3.1	2.8	1.8 - 4.8	3.0 ± 1.1	2.6 - 3.4	2.7	2.3 - 7.9	0.09	0.925
Cu	4.10 ± 0.98	3.40 - 4.80	3.86	3.21 - 6.39	3.51 ± 0.49	3.16 - 3.86	3.37	2.98 - 4.52	1.27	0.203
	6.87 ± 1.95	6.23 - 7.50	6.43	4.61 - 13.72	7.28 ± 3	6.31 - 8.25	6.64	4.41 - 20.45	0.54	0.591
Fe	233 ± 33	209 – 257	235	190 - 290	194 ± 32	171 - 217	195	150 - 250	2.07	*0.038*
	309 ± 58	290 – 327	310	210 - 400	332 ± 59	313 - 351	330	190 - 480	1.92	0.055
Hg	0.155 ± 0.018	0.142 - 0.168	0.150	0.136 - 0.200	0.118 ± 0.014	0.107 - 0.128	0.122	0.097 - 0.136	2.80	*0.005*
	0.137 ± 0.019	0.131 - 0.143	0.136	0.107 - 0.173	0.136 ± 0.023	0.128 - 0.143	0.131	0.100 - 0.188	0.59	0.558
K	2770 ± 221	2612 - 2928	2750	2500 - 3300	2720 ± 103	2646 - 2794	2700	2600 - 2900	0.41	0.683
	2100 ± 355	1985 - 2215	2100	1400 - 2800	646 ± 309	546 - 746	600	200 - 1800	5.44	*5.3×10-8*
Mg	727 ± 65	681 – 773	735	580 - 830	768 ± 121	681 - 855	785	600 - 930	0.97	0.333
	846 ± 120	807 - 885	830	620 - 1120	956 ± 156	905 - 1006	940	700 - 1400	3.79	*1.5×10-4*
Mn	94 ± 21	79 – 109	99	48 - 128	108 ± 34	84 - 132	116	65 - 173	1.07	0.285
	93 ± 24	86 - 101	93	45 - 148	119 ± 40	106 - 132	108	61 - 294	2.91	*0.004*
Mo	0.14 ± 0.02	0.12 - 0.15	0.14	0.11 - 0.17	0.13 ± 0.02	0.11 - 0.14	0.13	0.10 - 0.15	1.36	0.173
	0.21 ± 0.07	0.19 - 0.23	0.20	0.11 - 0.53	0.15 ± 0.07	0.13 - 0.17	0.12	0.09 - 0.49	4.76	*1.9×10-6*
Na	20 ± 0	-	20	20 - 20	22 ± 4	19 - 25	20	20 - 30	1.34	0.180
	15 ± 5	-	20	10 - 20	12 ± 4	11 - 13	10	10 - 20	2.48	*0.013*
Ni	0.8 ± 0.2	0.7 - 0.9	0.7	0.6 - 1.2	0.7 ± 0.1	0.6 - 0.8	0.7	0.6 - 0.9	1.40	0.161
	1.1 ± 0.4	1.0 - 1.3	1.0	0.7 - 2.6	1.2 ± 0.6	1.0 - 1.4	1.0	0.6 - 3.7	0.81	0.416
P	485 ± 115	402 – 568	475	370 - 770	454 ± 75	401 - 507	455	340 - 600	0.65	0.515
	477 ± 77	452 - 502	470	290 - 660	151 ± 76	126 - 176	140	100 - 590	5.43	*5.7×10-8*
Pb	1.84 ± 0.23	1.67 – 2.00	1.82	1.47 - 2.18	1.50 ± 0.17	1.38 - 1.62	1.49	1.30 - 1.86	2.19	*0.028*
	2.05 ± 0.32	1.95 - 2.16	1.97	1.49 - 2.93	2.22 ± 0.34	2.11 - 2.33	2.19	1.36 - 3.11	2.34	*0.019*
S	790 ± 208	641 - 939	800	500 - 1200	620 ± 123	532 - 708	600	500 - 800	1.58	0.114
	810 ± 139	765 - 855	800	500 - 1100	528 ± 89	499 - 557	500	500 - 1000	4.74	*2.1×10-6*
Sb	0.03 ± 0.01	0.02 - 0.03	0.02	0.02 - 0.04	0.02 ± 0.00	0.02 - 0.02	0.02	0.02 - 0.03	1.60	0.109
	0.05 ± 0.02	0.04 - 0.06	0.05	0.02 - 0.09	0.05 ± 0.02	0.04 - 0.05	0.05	0.02 - 0.11	1.10	0.271
Sr	14.6 ± 4.4	11.5 - 17.8	13.7	10.1 - 22.5	11.8 ± 2.7	9.8 - 13.8	11.5	7.9 - 17.4	1.48	0.139
	13.3 ± 2.9	12.4 - 14.2	13.0	8.6 - 23.7	14.7 ± 4.8	13.1 - 16.3	13.3	8.3 - 29	1.55	0.121
Ti	4.1 ± 0.7	4.0 – 5.0	4.0	3.0 – 5.0	3.3 ± 0.5	3.0 - 4.0	3.0	3.0 - 4.0	1.94	0.052
	5.1 ± 1.0	5.0 - 5.0	5.0	4.0 - 8.0	6.3 ± 0.9	5.0 - 6.0	6.0	3.0 - 7.0	1.73	0.083
Zn	20.9 ± 2.2	19.4 - 22.5	21.4	16.8 - 24.2	19.1 ± 1.9	17.8 - 20.4	19.3	15.7 - 21.6	1.38	0.169
	24.4 ± 3.2	23.3 - 25.4	24.2	19.4 - 31.6	25 ± 3.9	23.7 - 26.3	24.1	19.2 - 37.9	0.68	0.494

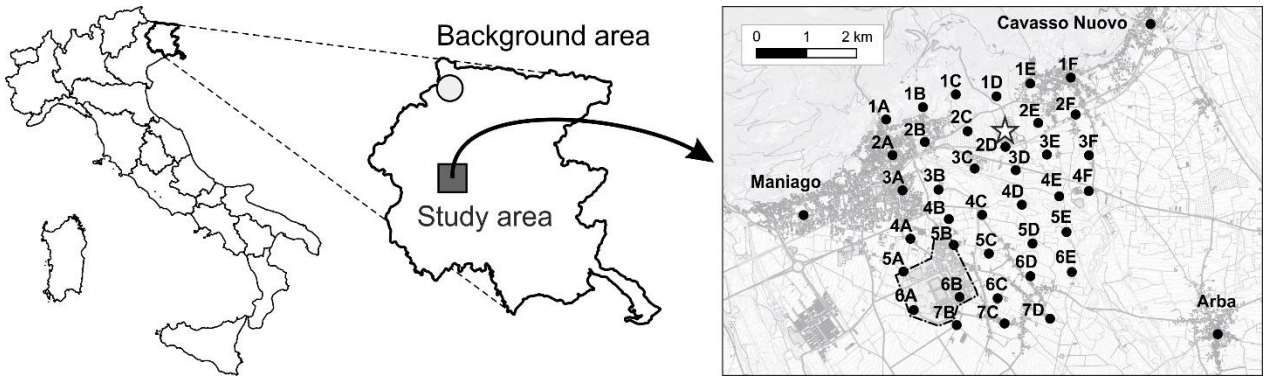
Supplementary Table S4. *EU* ratio, percentile-based, five-class interpretative scale for bioaccumulation data (i.e., Bioaccumulation Scale) from lichen transplants (Cecconi et al. 2019). Class codes, description and abbreviations, percentile thresholds, and the corresponding *EU* values associated with bioaccumulation classes are reported.

Bioaccumulation class			Percentile thresholds	*EU* ratio (8 weeks)
ID	Description (abbreviation)			
1	Absence of bioaccumulation	(A)	$\leq 25^{th}$	≤ 1.0
2	Low bioaccumulation	(L)	$(25^{th}, 75^{th}]$	(1.0, 1.9]
3	Moderate bioaccumulation	(M)	$(75^{th}, 90^{th}]$	(1.9, 2.7]
4	High bioaccumulation	(H)	$(90^{th}, 95^{th}]$	(2.7, 3.5]
5	Severe bioaccumulation	(S)	$> 95^{th}$	> 3.5

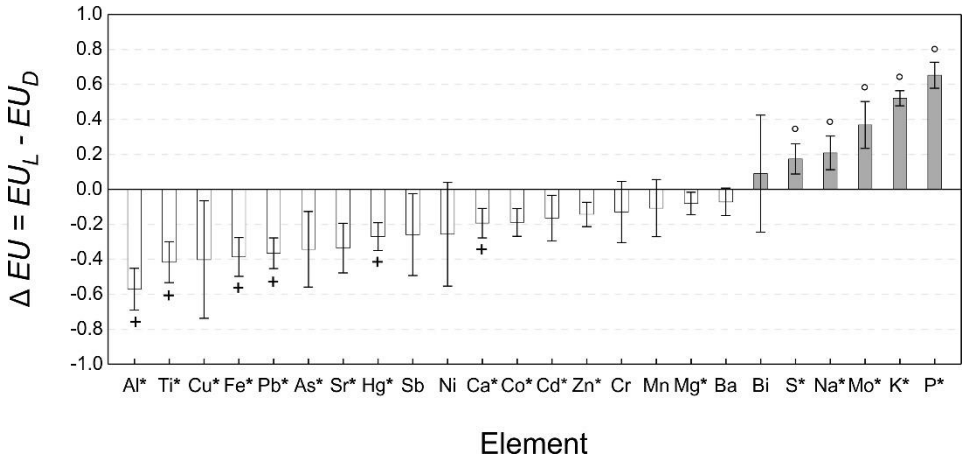
SUPPLEMENTARY FIGURES S1-S4



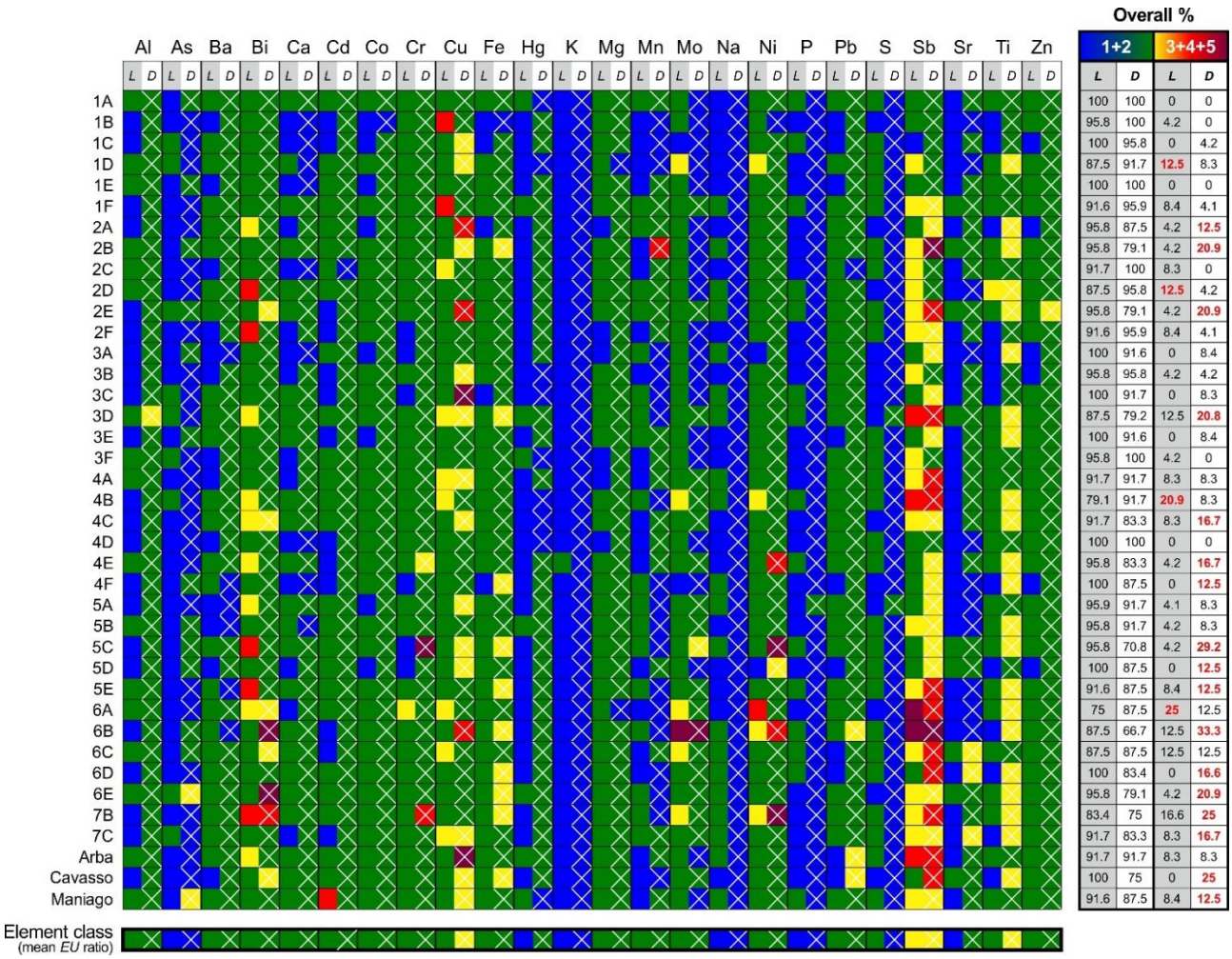
Supplementary Figure S1. Results of the post-storage Chl$_a$F assessment on unexposed and exposed sample sets (U_L: unexposed living samples, i.e. stored for 18 months at -20°C; U_D: unexposed devitalized samples, i.e. dark-stored at c. 10°C for 18 months; E_L: living samples exposed for 6 weeks in the study area; E_D: devitalized samples exposed for 6 weeks in the study area). Boxplots show median F_v/F_m values, interquartile ranges, minima and maxima, and outlier values (circles).



Supplementary Figure S2. Localization of the study area and the background area (for the collection of lichen samples), with indication of the transplant sites of *Pseudevernia furfuracea* labelled by alphanumeric codes (1A - 7D plus Arba, Cavasso Nuovo and Maniago). The two main putative pollution sources, a medium-sized cement plant and an industrial park, are respectively indicated by a star and a closed dashed line.



Supplementary Figure S3. Bar charts showing mean differences between *EU* ratio values of living (*L*) and dead (*D*) *P. furfuracea* samples (Δ *EU*) (error bars indicate 95% confidence intervals). Elements showing higher *EU* ratio values in more than 80% of transplant sites in either *L* or *D* samples are respectively marked with circles or crosses. Asterisks next to element labels indicate significant differences between the sample sets, according to the Wilcoxon signed rank test for paired samples (cf. Fig. 3 and Table 1).



Supplementary Figure S4. Classification of the transplant sites according to the analytical results in living (*L*, unmarked cells) and devitalized (*D*, cross-marked cells) samples, as resulting from the Bioaccumulation Scale for 8-week transplants (Supplementary Table S4). The element class, calculated by averaging *EU* ratios by column, is reported at the bottom of the figure (element class). The overall percent of elements in classes 1 and 2, as well as in classes 3, 4 and 5, is also reported site per site (limited to the sum of classes 3-5, percentages higher than 10% are highlighted in red).

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CONCLUSIONS

This research faces some non-negligible methodological aspects in bioaccumulation techniques. As with physicochemical methods, the quality of biomonitoring results depends on their reproducibility, which is enhanced by the fine tuning of interpretational tools, by minimizing the heterogeneity of sampling, processing and analytical procedures, and by increasing the knowledge on possible confounder phenomena affecting response variables. The results presented in this thesis aim to contribute to the advocated standardization of biomonitoring techniques.

As a first contribution, a large-scale outline of background element content (BEC) in *Pseudevernia furfuracea*, one of the most frequently used lichen bioaccumulator, is presented. Primarily, it was demonstrated that the BEC variability is almost unrelated to taxonomic traits at infraspecific level, with inter-varietal differences generally not overcoming the uncertainty associated to the analytical procedures, therefore leaving open the possibility of the joint use of the varieties in bioaccumulation applications. In this light, benchmark element content references could be provided without separately addressing the varieties of *P. furfuracea*.

The extensive review of active and passive bioaccumulation studies targeting this macrolichen revealed a huge methodological variability, especially concerning the sample processing and the mineralization step. However, the construction and the comparative analysis of methodologically homogeneous element concentration datasets allowed to provide a first overview of BEC ranges at supra-national level. Limited to original field data from Italy, species-specific, methodologically homogeneous BECs were provided for 43 elements and geographically separated contexts, to be used as reference datasets for biomonitoring applications.

P. furfuracea background levels were also explored after having performed a different mineralization of samples. BEC patterns assessed at very large scale proved to be rather conservative, irrespective the acid digestions, however, some interesting differences in spatial patterns arose. Therefore, a major methodological gap in biomonitoring procedures was filled by providing a complete set of reference values for one of the most used lichen biomonitors, based on different acid digestions, to be alternatively used according to the selected mineralization procedure.

Another step towards the standardization of bioaccumulation techniques was done by developing new interpretative tools for the assessment of the magnitude of pollution phenomena. Previously available interpretative scales for bioaccumulation results from native and transplanted lichens (based on very different assumptions) were popularly used in Italy and abroad. However, both scales were never critically revised, in spite of some overt flaws and the "aging" of bioaccumulation data on which previous scales for native lichens relied. By recovering core ideas from the old scales, new ones were developed, based on the meta-analysis of recent, methodologically consistent bioaccumulation data. These are based on the concept that pollution can be quantified by dimensionless ratios between experimental (i.e. concentrations measured in native or transplanted lichens) and benchmark values (BECs or pre-exposure values, respectively for native and transplanted lichens), therefore providing a harmonized framework for the two techniques.

Concerning "smaller scale" aspects possibly affecting the quality and the interpretation of bioaccumulation data, the investigation of the physiological response of *Pseudevernia furfuracea* to

complex pollutant mixtures proved the macrolichen to be a rather tolerant biomonitor, able to cope with stressing urban conditions. Field-stressed *P. furfuracea* even exhibited a photosynthetic recovery when ozonated, thus making the target species classifiable as fully tolerant to ozone.
Limited to samples exposed to high PAH levels, the peculiar physiological and PAH accumulation pattern after the ozone fumigation suggested that a significant decrease of PAHs in ozonated lichens may possibly be ascribed to the oxidative degradation of the most abundant category of such compounds. Although further investigation is needed to clarify this aspect, the possibility of an underestimation of PAH enrichment levels should seriously be taken into account when carrying out transplant-based surveys with contextually high ozone ground concentrations, as possibly altering bioaccumulation outcomes.
Finally, with respect to the effect of lichen vitality on the accumulative performance of the test species, the results presented in this thesis further corroborate the enhanced ability of dead lichen matrices in accumulating particulate-related elements. Overall, the contextual use of living and non-living *P. furfuracea* samples consistently described the severity of element deposition levels in the study area, however dead lichens show generally higher accumulation signals for lithogenic and environmentally-concerning elements. The interpretational discrepancies arising from the use of samples characterized by different physiological status pose the issue of sample devitalization in lichen biomonitoring.

To date the importance of biological monitoring is no longer questioned, as providing information that is by definition not achievable by physico-chemical methods. In this framework, the biomonitoring research has made great strides in the last years, raising biochemical, physiological and ecological questions as well as non-trivial statistical issues. Indeed, the production of reliable and highly robust results by using lichens (and organisms in general) depends on the proper assessment of the uncertainty associated to the target response variables, as well as on the understanding of phenomena underlying responses in complex systems, which certainly pose stimulating research challenges. As an example, deepening investigations are needed to clarify the role of lichen secondary metabolites on metal homeostasis and accumulation signals. Also, the effects of peculiar morphological features of thalli on their bioaccumulation capacity are still unquantified, although the variability in thallus architecture (e.g., size and branching) and the occurrence of peculiar reproductive structures (e.g., sexual and asexual reproductive propagules) certainly plays a role in particulate interception.
Besides the need to enhance our knowledge of the processes affecting bioaccumulation outcomes, at a broader level, the huge bioaccumulation data asset produced by the long-standing implementation of lichen biomonitoring should be valorised by developing effective and accessible data repositories and by keeping working on the methodological harmonization.

APPENDIX I

NEW ECOLOGICAL INSIGHTS FOR THE MACROLICHEN *PSEUDEVERNIA FURFURACEA*

PREMISE

The preliminary study presented in this Appendix was carried out as a corollary work during the three years of this PhD project. It does not directly deal with the biomonitoring of environmental pollutants, but it faces an aspect related to the monitoring of environmental changes by using a bioindication approach based on the response of a single lichen species. Indeed, in this study, some aspects related to the ecology of *Pseudevernia furfuracea*, the recurring figure of this work, are faced in view of providing a starting point to use its taxonomical varieties to highlight climate changes in clean-air environments. As frequently stated, the features of this species have made it a highly performing bioaccumulator. Nonetheless, it has also been used as bioindicator (either at the species or varietal level) through the assessment of its physiological response in semi-natural and urban environments (Tretiach et al. 2007; Pirintsos et al. 2011; Malaspina et al. 2018), but a missing piece consists in predicting varietal distributional shifts under different climate change scenarios. Despite the rapid improvement of niche modelling methods (Ellis et al. 2007), some important theoretical limitations still remain. Indeed, these approaches frequently suffer the lack of integration with the ecological theory, which is instead essential for a proper selection of causal environmental predictors (Pearson and Dawson 2004; Thuiller et al. 2004; Guisan and Thuiller 2005; Branquinho et al. 2015). In this respect, part of the solution may consist in basing predictions on fundamental (e.g., physiological) responses obtained from field or laboratory experiments, and constrain these by general rules of abiotic-biotic interactions and dispersal behavior, in order to obtain more realistic predictions of taxa distribution under changing environments (Evans et al. 2015). This still ongoing study, "*Infraspecific physiological response of the macrolichen Pseudevernia furfuracea to environmental changes: An altitudinal transplant experiment in a clean-air region of the Alps*", fits into this context. In light of a multi-marker approach consistent with that used in the previously presented works, and the focus on this species as biomonitor of environmental changes, it was thought not to sin in including such preliminary results as an Appendix, with the hope that these could be of some interest, waiting for their better formalization.

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Infraspecific physiological response of the macrolichen *Pseudevernia furfuracea* to environmental changes: An altitudinal transplant experiment in a clean-air region of the Alps

Preliminary draft

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Abstract

The montane epiphytic macrolichen *Pseudevernia furfuracea* consists of two morphologically identical varieties, var. *furfuracea* and var. *ceratea*, altitudinally vicariant in the Alps. The aim of this work is to investigate the intervarietal physiological response to environmental changes in clean-air environments, testing the hypothesis that the altitudinal distribution of varieties is driven by microclimatic and climatic factors.

Thalli of the two varieties were collected at treeline sites in three areas of the southeastern Alps along a precipitation gradient, and transplanted for six months at c. 1000 m. Within each area, four transplant sites characterized by different microclimatic conditions were selected, and the response to transplant was assessed by means of chlorophyll fluorescence emission (Chl_aF), malondialdehyde (MDA) and ergosterol (ERG) content. After six months, Chl_aF decreased, without significant intervarietal differences. By contrast, peculiar intervarietal variations were highlighted for MDA and ERG content, reflecting the major role of the mycobiont. Unexpextedly, MDA content decreased, suggesting that the environmental oxidative stress was lower at 1000 m than at the treeline. However, var. *ceratea* showed significantly higher MDA levels, especially at the wettest sites. By contrast, the ERG content generally lowered after the transplantation, indicating a lichen recovery. However, in this case, consistently with MDA patterns at the transplant sites, var. *ceratea* exhibited lower ERG levels at two out of three areas. The two fungal markers were substantially correlated with the relative humidity at the transplant sites, however the biochemical mechanisms underlying such response still need to be clarified.

Data analyses, as well as the characterization of secondary metabolite profiles of varieties, and their biotic interactions with lichenivorous gastropods (land slugs), are still in progress.

Keywords: lichen varieties, environmental drivers, *ceratea*, biomonitoring, chlorophyll fluorescence, malondialdehyde, ergosterol, lichen substances.

1. Introduction

Lichens are reliably used to monitor a wide range of atmosheric changes, ranging from pollutant and contaminants over space and time (e.g., Tretiach et al. 2011; Pinho et al. 2011) to climatic changes (e.g., Ellis et al. 2007; Matos et al. 2017). The great suitability of these organisms depends on several features, such as (*i*) their ubiquity over terrestrial environments, (*ii*) their perennial nature, (*iii*) the lack of seasonality, (*iv*) their poikilohydry (which determines the strong link with the atmospheric compartment), (*v*) their independence from soil for mineral nutrition, and (vi) the good poleotolerance of several species (Ellis et al. 2007), implying the possibility of minimizing complex confounder effects.

The assessment of the spatial distributions of lichen species and their variations caused by major environmental drivers, provides interesting autoecological information on symbiotic organisms and, under an application perspective, it is an effective tool to highlight the effects of environmental changes (Branquinho et al. 2015). A possible approach is the implementation of species distribution models, which are widely used to correlate species landscape distributions with macroclimate, projecting and comparing suitable climate space at a baseline and for future climate change scenarios (Ellis et al. 2017). This approach relies on the simplistic assumption that species distributions are at equilibrium with, and directly sensitive to macroclimate (Guisan and Thuiller 2005). Although for lichens such an assumption is less problematic than for other taxonomic groups, these techniques still suffer important theoretical limitations, due to the fact that the response of many taxa to climate change (including lichens) can be influenced by prevailing smaller-scale factors, such as spatial habitat patterns (Selva 1994; Kuusinen 1996), dispersal ability (Travis 2003; Iverson et al. 2004), and the potential influence of pollution phenomena (Branquinho et al. 2015). Moreover, the lack of integration of models with the ecological theory poses additional issues (Austin 2002; Wiens 2002; Guisan and Thuiller 2005), leading to the failure of identification of causal environmental predictors (Pearson and Dawson 2004; Thuiller et al. 2004). In this respect, part of the solution consists in basing predictions on fundamental (e.g., physiological) responses obtained from field or laboratory experiments, and constrain these by general rules of biotic interactions and dispersal behaviour (Evans et al. 2015).

Another major aspect is the thorough choice of spatial scales of the study domain and the target taxa. The well-known macrolichen *Pseudevernia furfuracea* (L.) Zopf well fits to this purpose. *P. furfuracea* is a fruticose, meso-xerophilous, photophilous species (Rikkinen 1997), with a cool-temperate to boreal-montane distribution (Smith et al. 2009). In Italy the species is abundant in montane and subalpine forests and it is widely used as a bioaccumulator in passive and active biomonitoring applications (e.g., Nascimbene et al. 2014; Kodnik et al. 2017).

Interestingly, *P. furfuracea* has four chemical strains grouped into taxonomical varieties with different secondary metabolite profile and distributions: (*i*) *P. furfuracea* var. *furfuracea* and (*ii*) *P. furfuracea* var. *ceratea*. In the 1950s, Mason E. Hale, based on thousands of herbarium and museum specimens, analysed the relative occurrence of *P. furfuracea* strains at 1911 sites in Europe, finding a general pattern of increasing frequency of variety *ceratea* going northwards, ranging from the absence of var. *ceratea* in North Africa to its predominance in Great Britain (with c. 80% of samples; Hale 1956). The author was also the first to hypothesize that the occurrence of

the varieties (as well as the synthesis of lichen substances) could be driven by climatic gradients. To date, several further studies reporting the relative frequency of the varieties / strains in European regions are available (Moruzi and Cucu 1969; Nil'son 1970; Hawksworth and Chapman 1971; Halvorsen and Bendiksen 1982; Redondo and Reol 1989; Martellos 2003; Tretiach et al. 2013), but the correlative nature and the complex response of symbiotic oganisms to environmental conditions did not allow to identify the distributional abiotic drivers.

Interestingly, in the Alps, *P. furfuracea* varieties exhibit a clear altitudinal vicariance, with the frequency of var. *ceratea* increasing with altitude, reaching a maximum at timberline sites, although with marked differences between western and eastern Alps (Tretiach et al. 2013). In particular, var. *ceratea* has its optimum at 1600-1800 m a.s.l., whereas var. *furfuracea* is more common in the beech belt, apparently under wetter climatic conditions (Martellos 2003). Even in this context, the role of abiotic drivers (e.g. precipitation, temperature and humidity) is still unclear (Martellos 2003; Tretiach et al. 2013), so as the occurrence of possible biotic interactions with niche-defining effects.

The peculiar Alpine distribution of the varieties poses interesting possibilities, since climatic factors (especially temperature) show similar trends with elevation and latitude, allowing to assume that altitude gradients may be used as downscaled proxies for understanding ecological processes along latitudinal gradients (Halbritter et al. 2013). Moreover, the abundance of the species, its good dispersal ability, and the lack of substantial influence of pollution on its distribution, further contribute to the minimization of confounding factors, therefore making it an optimal candidate to highlight environmental changes in clean-air contexts.

Main aim of this study is to assess the infraspecific physiological response of the two taxonomical varieties of *P. furfuracea* to changes in environmental conditions using the transplant technique. Inherent objectives are (*i*) to identify the main abiotic drivers for the distribution of the lichen varieties, and (*ii*) to possibly assess whether other abiotic or biotic factors may influence their distribution.

2. Materials and methods

2.1 The target species: taxonomy, chemical strains and varieties

Four strains of *Pseudevernia furfuracea* (L.) Zop have been recognised (Ferencova et al. 2010). These, differing for the secondary metabolite profile, are morphologically identical. Chemical race I contains physodic and oxyphysodic acids, chemical race II contains physodic and olivetoric acids, chemical race III has only olivetoric acid, and chemical race IV has olivetoric, physodic and oxyphysodic acids.

Pseudevernia furfuracea was treated by earlier authors (e.g., Hale 1956, 1968) as a three-species complex: the isidiate *P. furfuracea* (L.) Zopf, with physodic and oxyphysodic acids; the isidiate *P. olivetorina*, with olivetoric acid; *P. soralifera* (Bitt.) Zopf, with physodic and oxyphysodic acids and with soralia in addition to isidia. Several authors do not support Hale's separation at species rank of *P. furfuracea, P. olivetorina* and *P. soralifera*, and suggest to consider them as varieties (e. g., Poelt 1969; Hawksworth and Chapman 1971; Hafellner and Obermayer 2004). Indeed, in the most recent literature they are distinguished at the varietal level (e.g., Martellos 2003; Tretiach et al. 2013): *P. f.* var. *furfuracea* (L.) Zopf (the nominal one) and *P. f.* var. *ceratea* (Ach.) Hawksw. Var.

furfuracea has olivetoric, physodic and oxyphysodic acids as main medullary compounds and corresponds to chemical race I. Var. *ceratea* may produce physodic and/or oxyphysodic acid or neither, but it always produces olivetoric acid (its diagnostic metabolite), thus corresponding to chemical races II, III and IV (Supplementary Methods S1.1; Supplementary Fig. S1).

The presence of olivetoric acid in var. *ceratea* allows to distinguish the varieties with a simple spot test with diluted sodium hypochlorite applied to the medulla (C-test; Culberson 1965; Elix and Stocker-Wörgötter 2008). The olivetoric acid, reacting with the sodium hypochlorite, causes a positive colour reaction (C+; from pink to bright red) in thalli of var. *ceratea*, while a negative reaction (C-) occurs in var. *furfuracea*.

2.2 Experimental design

Thalli of the two varieties of *Pseudevernia furfuracea* were collected at treeline sites and transplanted at lower altitudes. The transplant experiment was replicated in three areas (A, B, C; Table 1; Fig. 1) of the south-eastern Alps, along a longitudinal transect corresponding to a clear climatic gradient of mean annual precipitation (Fig. 1; Supplementary Methods S1.2; Supplementary Fig. S2).

Table 1. List of field sites with indication of the study area (A, B, C), site type (*c*: collection site; t: transplant site), identification codes (as in Fig. 1), altitude, orientation of the slope, presence of lentic water bodies (lake), geographical coordinates (DD; decimal degress) and tree species (either the phorophyte for the collection site or the tree species selected for the lichen exposure at the transplant sites).

Area	Site type	Site ID	Altitude (m a.s.l.)	Slope orientation	Locality name (adm. province)	Lake	Coordinates (DD) (East, North)	Phorophyte * / transplant tree
	c	A	1960	S/SW	Val Ombretta (BL)	-	11.869492, 46.424171	*Larix decidua* *
	t	A1	1010	N/NE	Alleghe (BL)	Alleghe	12.010854, 46.411709	*Acer pseudoplatanus*
A	t	A2	1030	SE	Alleghe (BL)	Alleghe	12.010119, 46.400268	*Picea abies*
	t	A3	1090	N/NE	Celat (BL)	-	11.941807, 46.359001	*Fagus sylvatica*
	t	A4	1075	S/SE	Carfon (BL)	-	11.922326, 46.365508	*Acer pseudoplatanus*
	c	B	1830	S/SE	Sella di Rioda (UD)	-	12.638272, 46.474482	*Larix decidua* *
	t	B1	1000	N/NW	Sauris lake (UD)	Sauris	12.719872, 46.444946	*Picea abies*
B	t	B2	985	S/SW	Sauris lake (UD)	Sauris	12.727240, 46.451918	*Acer pseudoplatanus*
	t	B3	1005	N/NE	Passo di Monte Rest (UD)	-	12.782370, 46.360637	*Fagus sylvatica*
	t	B4	960	S	Forni di Sotto (UD)	-	12.651387, 46.401179	*Fagus sylvatica*
	c	C	1800	S/SW	Mt. Lussari (UD)	-	13.528037, 46.470977	*Larix decidua* *
	t	C1	970	NW	Predil lake (UD)	Predil	13.562843, 46.415320	*Picea abies*
C	t	C2	975	S/SE	Predil lake (UD)	Predil	13.564817, 46.424116	*Fagus sylvatica*
	t	C3	960	NW	Riofreddo valley (UD)	-	13.561053, 46.467803	*Picea abies*
	t	C4	1005	S/SW	Strmec na Predelu (Bovec)	-	13.610071, 46.417435	*Fagus sylvatica*

Source lichen populations were selected immediately below the treeline (1800 - 1960 m a.s.l.; Table 1), corresponding to the upper altitudinal limit for the species (Martellos 2003). All the collection sites were located along south-oriented slopes with open larch stands (*Larix decidua* Mill.) in order to minimize the accessory variability related to different native ecological conditions (Elzinga et al. 2001).

Within each area, four sites were selected for the transplantation (henceforth, transplant sites). Transplant sites were located at lower altitudes (960 - 1090 m a.s.l.), where the occurrence of

natural populations of the nominal variety was previously assessed. These sites were characterized by different slope orientation (north or south) and by the presence/absence of a lake (Fig. 1, Table 1), in order to ensure different microclimatic conditions.

The transplant lasted 6 months, from early December 2017 to early June 2018. During this period, microclimatic conditions (temperature, relative humidity and dew point) were monitored at the study sites. At the end of the exposure, thalli were retrieved, processed as described in Sect. (2.2) and subjected to the determination of physiological markers (Sect. 2.3).

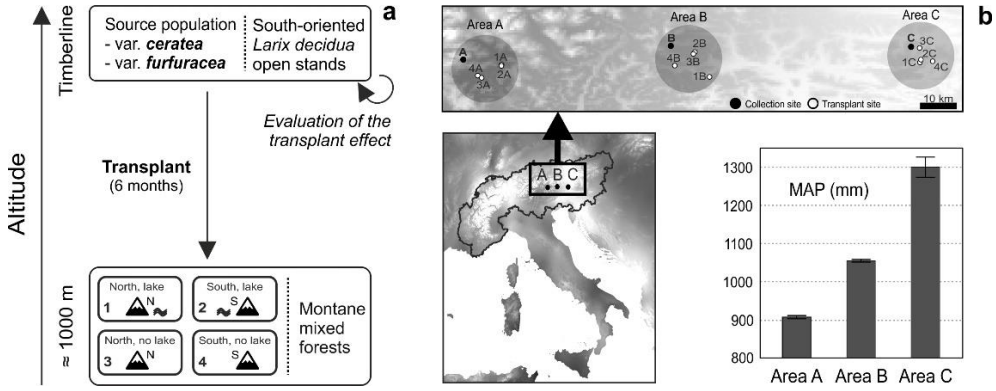

Figure 1. Schematic representation of the experimental design (a) and geographic location of the study sites (including collection and transplant sites), along with a bar chart showing mean annual precipitation and associated standard deviations (MAP) of areas A, B and C (b).

Besides the transplantation of samples at lower altitudes, a suitable number of lichen thalli was sampled at each collection site, directly sorted in the field according to the variety, mounted on exposure devices (Sect. 2.2) and transplanted on larch branches in order to assess the potential effect caused by the transplant itself (Fig. 1; Supplementary Methods S1.3, Supplementary Results S1.1).

2.2. Lichen collection, processing, exposure and retrieving

Thalli of *P. furfuracea* var. *furfuracea* and var. *ceratea* were collected at the three clean-air treeline sites in October 2017. At each site, a minimum of 250 lichen-carrying tweegs were sampled from larches. The lichen material was transported to the laboratory, where thalli were left to dry out at room temperature. Lichens were cleaned from debris, fragments of tree bark, and other extraneous material, and then subjected to a careful sorting according to the infraspecific variety by repeated C spot tests (Sect. 2.1), taking extreme care in the subdivision of intermingled thalli. Thin layer chromatography (TLC) was also performed on a small set of randomly selected thalli to confirm the efficacy of the spot test and to preliminarily assess the chemical strains of var. *ceratea* (methodology and an example of chromatographic outcome is reported in Supplementary Methods S1.1 and Supplementary Fig. S1).

Approximately 120 thalli of each variety were randomly selected from the bulk for each collection site (A, B, and C): c. 60% of the material (72 thalli) was chosen for transplantation, whereas c. 40% was used to assemble the experimental samples for the pre-exposure assessment of MDA and ergoterol (destructive measurements).

Pre-exposure measurements of chlorophyll *a* fluorescence were carried out on the same lichens intended to be transplanted (72 measurements per collection site and variety, overall 432 measurements), whereas pre-exposure measurements of MDA and ergosterol were performed on eight samples per variety and site.
For each area, 72 thalli were randomly subdivided in four groups for the transplantation, ensuring that no significant differences occurred among groups in terms of fluorescence parameters.
A week before the exposure, lichen thalli were mounted on exposure devices. Thalli, still attached on their twigs, were secured with plastic bonds to wooden rods (120 cm long, 0.5 cm Ø) and carefully covered with a soft, clean plastic net (12 mm mesh) to avoid the massive loss of lichen material due to accidental events in the field. Immediately after their preparation, on early December 2017, paired exposure devices carrying samples of the two varieties were placed at each transplant site within 7 days of field work. At each site, four exposure devices (two per variety) were attached to tree branches at approximately 3 - 4 m above the ground.

At early June, after 6 months of exposure, samples were retrieved within 7 days of field work and transported to the laboratory, where these were processed according to Sect. 2.3 for the determination of physiological markers. In this case, 18 Chl$_a$F measurements and five MDA / ergosterol content measurements were carried out on samples of each variety per transplant site. Overall, 432 measurements were carried out for Chl$_a$F, and 120 for MDA and ergosterol, respectively.

At the moment of their retrieving and immediately afterwards in the laboratory, lichen samples were visually analysed to assess potential biomass loss, mechanical damage, thallus bleaching, as well as the occurrence of small arthropods and moulds. In particular, mould-colonised thalli were discarded for MDA and ergosterol determination.

2.3 Physiological measurements

Three physiological markers were selected in order to encompass both symbiotic partners (Cecconi et al. 2019). In particular, chlorophyll *a* fluorescence (Chl$_a$F), malondialdehyde (MDA) and ergosterol (ERG) contents were respectively selected as proxies of the photosynthetic efficiency of the photobiont (Tretiach et al. 2007), peroxidation of membrane lipids (Candotto Carniel et al. 2017), and basal repsiration rates of lichens (Sundberg et al. 1999; Dahlman et al. 2002).

2.3.1 Chlorophyll a fluorescence

Chl$_a$F was assessed in terms of the maximum quantum yield of primary photochemistry in dark adapted samples (F_v/F_m). Chl$_a$F measurements were carried out on pre-exposure and transplanted thalli (post-exposure) with a Photosynthetic Efficiency Analyzer fluorimeter Handy-PEA (Hansatech, King's Lynn, UK). Measurements were carried out on scarcely isidiate terminal lobes of 2.5 cm length (Tretiach et al. 2005) after a preconditioning period of 48 hours in wet chambers with low light (5 μmolγ m^{-1} s^{-1}) (Gauslaa and Solhaug 2004), and after 30 minutes of dark-adaptation. Preconditioning of lobes was carried out in jars at c. 100% relative humidity (RH) for 48 h (PPFD, 30 μmol photons m^{-2} s^{-1}; 18 °C; 10 h dark / 14 h light). In order to ensure uniform environmental conditions during the hydration step, thermo-hygrometric sensors (EL-USB-1, lascar

Electronics, UK) were placed inside the jars to record temperature and RH every 30 minutes. Once hydrated at 100% RH, lobes were rinsed for 3 minutes in dH_2O, then gently shaken to remove the excess water, and dark-adapted for 30 min.
Post-exposure fluorescence measurements were carried out on the same thalli tested before exposure (18 measurements per site and variety, 144 measurements per area).

2.3.2 Malondialdehyde (MDA) assay

MDA is formed through auto-oxidation and enzymatic degradation of polyunsaturated fatty acids in membranes and reacts with two molecules of thiobarbituric acid (TCA) via an acid-catalyzed nucleophilic addition forming an orange compound with a maximum absorbance of 532 nm. Lipid peroxidation of *Pseudevernia furfuracea* varieties was determined by the thiobarbituric acid reactive substances (TBARS) assay, according to Candotto Carniel et al. (2017), in turn based on Heath and Packer (1968).

Samples of c. 200 mg each were grinded with liquid nitrogen, lyophilized, homogenized in a mortar using 1.5 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 12000*g* for 20 minutes at room temperature. An aliquot of 0.5 mL of the supernatant was collected and mixed with 1 mL of 20% TCA with 0.5% thiobarbituric acid (TBA). The mixture was heated at 95 °C for 25 minutes, quickly cooled in an ice bath and centrifuged at 15000*g* for 10 minutes at room temperature. The supernatant was removed and used to determine MDA concentration. Absorbance readings were taken at 532 nm using an UV-vis spectrophotometer (Jenway 6505, Stone, UK) and corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. The amount of MDA was calculated by using a molar extinction coefficient of 155 mM^{-1} cm^{-1} and the results expressed as nmol g^{-1} (DW).

2.3.3 Ergosterol

The content of ergosterol was determined according to protocol of Dahlman et al. (2002), slightly modified. Samples of c. 50 mg each were grinded with liquid nitrogen, lyophilized, and homogenized for 10 min in 96% ethanol. Extracts were transferred to 1.5 ml microtubes and shaken in the dark at 25 °C for 30 min, then vortexed and centrifuged at 10000 g for 20 min. The resulting supernatant was immediately analyzed by HPLC (Ultimate 3000 Pump, UVD170U UV-VIS detector, Dionex, Sunnyvale, CA, USA) in a reverse-phase Dionex column (Acclaim 120, C18, 5 µm particle size, 4.6 mm internal diameter × 150 mm length) as separator, with flow rate 0.8 ml/min and isocratic elution with methanol as mobile phase (Dahlman et al. 2002). Total analysis time was 15 min. Absorbance at 280 nm was measured with a UV detector (Dionex UV/Detector Lamps). A standard curve was prepared with 0.1-10 mg ergosterol from Sigma-Aldrich (USA) dissolved in 1 ml of ethanol. Data were extrapolated by Chromeleon software version 7 (Dionex Corporation, Sunnyvale, CA, USA), and results were expressed as µg mg^{-1} (DW).

2.4 Preliminary data analysis

Preliminarily, Spearman's ranks correlations coefficients were calculated and multivariate assessments were carried out on experimental data to remove redundant climatic variables for subsequent analyses (Supplementary Methods S1.2).

Descriptive statistics were calculated for mean annual precipitation (MAP) and microclimatic variables (T, RH, and DP) as well as for physiological response variables (F_v/F_m, MDA and ergosterol). Significant differences in terms of microclimatic conditions among the transplant sites were tested by Kruskal-Wallis ANOVA and Dunn's post hoc test (Supplementary Fig. S3).

Data, in the matrix form '12 transplanted samples × 3 physiological variables plus MAP, T, RH and DP', were explored by Principal Component Analysis (PCA). Lichen varieties and site-specific features (i.e., north/south slope exposure and the presence/absence of a lake) were included in the PCA as supplementary variables, i.e. plotted in the multivariate space, but not used to calculate the principal components (Legendre and Legendre 1998).

The median values of physiological markers in transplanted samples were tested for significant differences with respect to the corresponding pre-exposure values using the non-parametric Mann-Whitney test for independent samples, whereas intervarietal significant differences of physiological parameters at the transplant sites were tested using Wilcoxon test for matched paired samples.

Preliminary data analyses and graphics were performed with the software packages Statistica v. 10 (StatSoft Inc., Tulsa, OK, USA), R (R Core Team 2013) and Excel 2010 (Microsoft). Statistical significance was tested at $\alpha = 0.05$ in all cases.

3. Preliminary results

3.1 Microclimatic characterization of the exposure sites

Microclimatic data showed a rather expected pattern. Within each area, the lowest and highest air temperatures were registered respectively in north-exposed sites close to lakes (sites A1, B1, C1) and in south-exposed sites without nearby water bodies (A4, B4, C4). Overall, the coldest and warmest sites were B1 (3.1 ± 6.7 °C) and B4 (5.5 ± 7.3 °C) (Supplementary Fig. S3). The within-area pattern for relative humidity was expectedly specular to that of temperatures. Overall, the wettest and driest sites were B1 (87.4 ± 11.2%) and A4 (75.2 ± 18.2 %) (Supplementary Fig. S3). Monthly patterns of T and RH were rather conserved (data not shown). Regarding the dew point, the pattern was less conserved: within each area, the highest values were revealed at sites A1, B3, and C4, whereas the lowest were revealed at sites A4, B1 and C2 and overall, sites with highest and lowest DP were C4 (1.8 ± 6.6 °C) and A4 (0.6 ± 7.1 °C) (Supplementary Fig. S3). However, unlike T and RH, inter-site differences were rarely significant, a pattern also recurring monthly.

3.2 Infraspecific physiological response

The first two components of the PCA (PC 1 and PC 2) satisfactorily represent the multivariate structure of the data matrix, explaining 63.1% of the total variance. The multivariate exploration revealed a pattern of positive association among MDA, relative humidity (RH), and ergosterol (ERG), positively correlated to PC1, as well as var. *ceratea* (although the lichen varieties only showed weak correlations with both axes, especially with PC 2). F_v/F_m was instead negatively

associated to PC 2, so as the dew point (DP) (Fig. 2). A slight positive association was also revealed between F_v/F_m and the nominal variety.

The PCA consistently described the microclimatic pattern of the transplant sites (Sect. 3.1), highlighting positive associations amongst temperatures, south-exposed slopes and absence of lentic water bodies, as well as amongst relative humidity, north-exposed slopes and the presence of a lake. The ergosterol and the only climatic variable included in the analysis (MAP) showed substantial positive and negative associations with PC 1 and PC 2, respectively

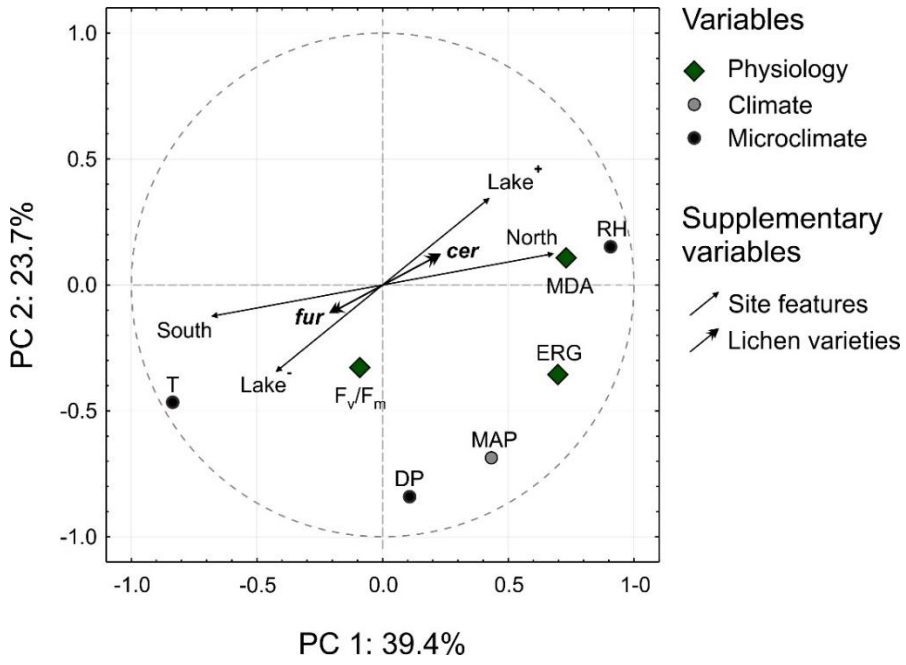

Figure 2. PCA of environmental variables and physiological response variables in *Pseudevernia furfuracea* samples transplanted at 12 field sites and their relationship with site features (slope orientation: north / south; presence / absence of a lake: lake$^+$ / lake $^-$) and lichen variety (*fur*: var. *furfuracea*; *cer*: var. *ceratea*).

Before the transplant, chlorophyll fluorescence (F_v/F_m), malondialdehyde (MDA) and ergosterol (ERG) median alues were significantly different in *P. furfuracea* var. *furfuracea* and *ceratea* at each collection site, with the exception of MDA values at site A (Fig. 3). F_v/F_m and MDA differences in paired samples at collection sites showed higher values in either variety, but this was not the case of ergosterol, always showing higher levels in variety *ceratea* (Fig. 3).

After the 6 month exposure, physiological parameters measured in transplanted lichen samples exhibited a pattern of variation with respect to pre-exposure values. Chl$_a$F always significantly decreased in transplanted samples, irrespective the lichen variety ($p < 0.05$; Mann-Whitney test; data not shown). Although post-exposure median values of F_v/F_m were significantly lower than pre-exposure ones, these were highly variable, ranging from 0.029 to 0.750, with only 11.3% of individual records below 0.400. Even MDA values significantly decreased in transplanted samples, with the exception of var. *ceratea* transplanted at north-exposed sites of areas A and C (i.e., A1, A3, C1, C3) and var. *furfuracea* transplanted at site C1, which did not show significant variations ($p > 0.05$; Mann-Whitney test; Fig. 3). Concerning ERG values, these consistently and significantly decreased for both varieties at each area, except var. *furfuracea* sample transplanted at site C1, that instead significantly increased ($p < 0.05$; Mann-Whitney test; Fig. 3).

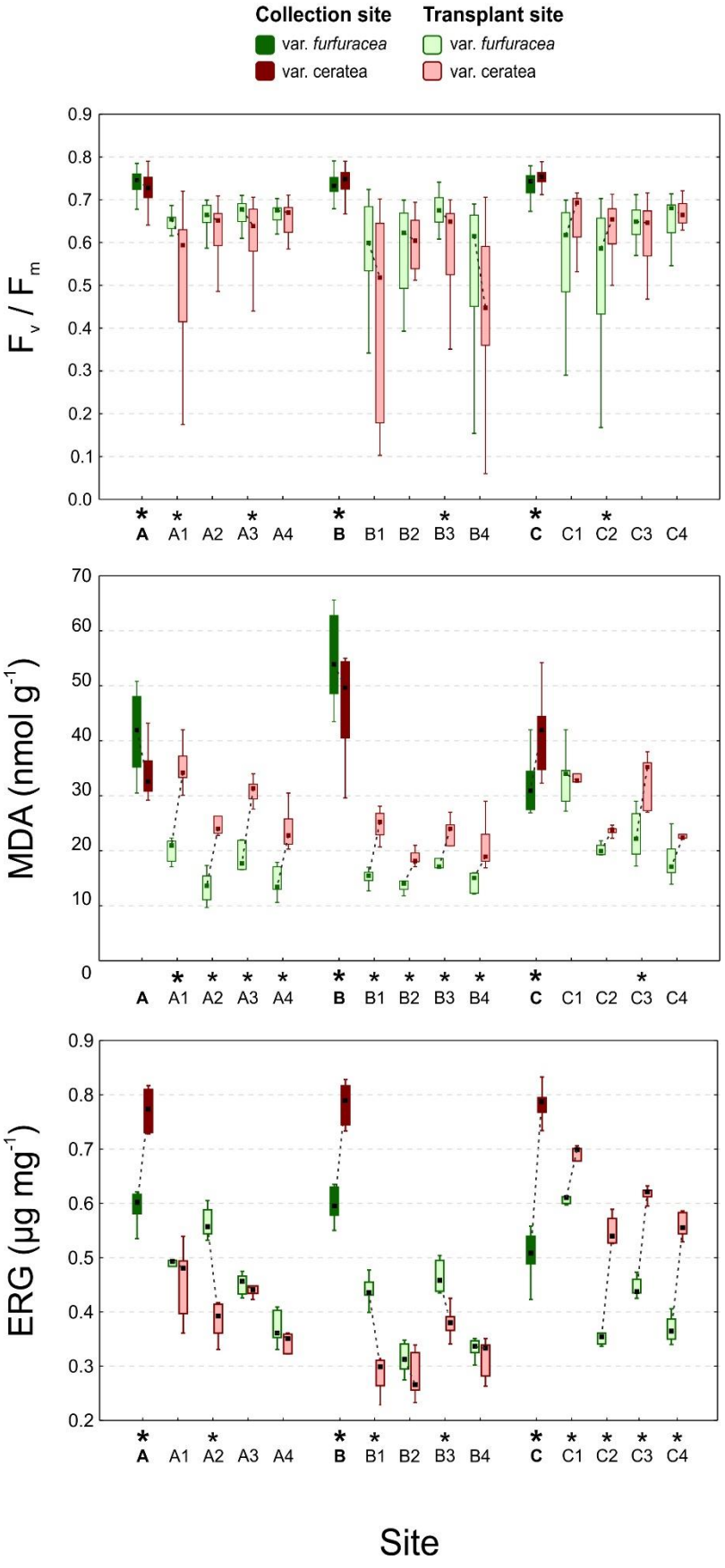

Figure 3. Boxplot representing median values (dots), interquartile ranges (boxes), and non-outlier ranges (whiskers) of physiological parameters (chlorophyll *a* fluorescence, F_v/F_m; malondialdehyde, MDA; ergosterol, ERG) in samples of the two varieties, before (A, B, C: collection sites) and after the exposure (A1-A4, B1-B4, C1-C4: transplant sites). Asterisks above site labels refer to intervarietal significant differences ($p < 0.05$), as highlighted by the Wilcoxon's matched paired test (Sect. 2.4).

When addressing paired samples of the two varieties at the transplant sites, F_v/F_m was significantly higher in the nominal variety limited to the sites A1, A3 and B3. Moreover, slightly but non-significantly higher values were also revealed in the nominal variety at sites A2, B1, B2, B3, C3 and C4. Significantly higher values were instead revealed in var. *ceratea* limited to site C2. By contrast, MDA was significantly lower in var. *furfuracea* at all transplant sites of areas A and B, and limited to site C3, whereas the opposite was never highlighted (Fig. 3).
The intervarietal pattern of ERG at the transplant sites was consistent in areas A and B, where the nominal variety showed always higher median values (significantly at sites A2, B1 and B3). Differently, at the transplant sites of area C, the situation was the opposite, with var. *ceratea* always exhibiting significantly higher median values (Fig. 3).

3.2 Grazing damage

At the moment of their retrieving, lichen samples resulted variably affected by the transplantation. The utmost damage was revealed in both varieties transplanted at sites A1, A3, and in var. *ceratea* transplanted at sites B1, and B4. These samples suffered the effects of arthropods grazing: in particular, land slugs were directly caught on transplanted thalli or alternatively, typical radula marks, faeces and slime silvery tracks were found on lichens and twigs. The slug was identified as *Lehmannia marginata* Müller (Supplementary Fig. S3), a typical lichenivory woodland species (Asplund et al. 2010) of the family Limacidae. *L. marginata* grazing determined significant sample loss due to mechanical damage and subsequent thallus fragmentation. Fortunately, enough lichen material was recovered to perform all the planned physiological measurements. Grazed samples exhibited greater fragility compared to non-grazed ones, also resulting substantially darkened and sporadically colonized by different unidentified moulds.

4. Preliminary discussion

4.1 Physiological response of *P. furfuracea* varieties to altitudinal transplants

After 6 months of exposure, *P. furfuracea* varieties showed a peculiar physiological pattern. As expected, the photosynthetic efficiency of photosystem II generally dropped, albeit not always significantly. The within-site variability of F_v/F_m at the transplant sites was on average higher than one order of magnitude compared to the pre-exposure one (standard deviations ranged between 0.028 and 0.231 in transplanted samples and between 0.021 and 0.041 in pre-exposure ones; Fig. 3). This is not surprising since *P. furfuracea* is considered a meso-xerophilous macrolichen (Smith et al. 2009), from mildly to extremely photophilous, according to its native ecological context (Lipnicki et al. 2012). In this study, *P. furfuracea* source populations were selected at south-oriented treeline sites on open stands of *L. decidua* to minimize accessory variability. However, such native conditions determine high mean photosynthetic photon flux densities, leading to the adaptation of photosynthetic levels to site-specific seasonal variations of irradiance, certainly different from those typical of mixed forests at lower elevations (Kershaw 1985). Given that, it is reasonable to assume that short-term changes in light availability, such as abrupt alterations in light exposure imposed by the transplantation of lichens, may result in their photoinhibition (Demmig-Adams et al. 1990; Gauslaa and Solhaug 1996), as also confirmed by the lowest variability

associated to F_v/F_m in samples exposed at south-oriented open stands of sites A2, A4, C4, and partially of site B2 (Fig. 3).

When addressing the infraspecific response in terms of Chl$_a$F, although F_v/F_m values were generally higher in transplanted *P. furfuracea* var. *furfuracea* samples, the impairment of the photosynthetic apparatus of algal populations did not substantially differ in the two varieties. Such results were different from those of Malaspina et al. (2018), which highlighted a different Chl$_a$F response to pollutant gradients of *P. furfuracea* varieties exposed at urban environments, indirectly confirming the absence of significant long-range transported pollutants at our transplant sites.

As far as MDA content, an unexpected pattern emerged. Indeed, MDA values were significantly lower than the corresponding pre-exposure values in the majority of cases and, unlike Chl$_a$F, even the within-site data variability was lower than that measured immediately after the collection at the timberline (Fig. 3). This was quite surprising, since, in absence of a significant amount of literature to perform inter-study comparison of data, we aprioristically assumed that the oxidative stress experienced by lichens in native, and presumably optimal conditions, would have been lower than that at suitable but non-native environmental contexts, as revealed in *P. furfuracea* var. *furfuracea* transplants reported by Lucadamo et al. (2015) and Cecconi et al. (2019).

A single work reported MDA content in *P. furfuracea* samples (irrespective the variety) along an altitudinal transect in the central-eastern Alps (Austria, Lungau district; Schlee et al. 1995). Schlee et al. (1995) analysed MDA content and superoxide dismutase activity (SOD) in samples collected at seven sites between 1080 and 1940 m a.s.l. These authors related the MDA content to total soluble protein content or to the fresh weight (FW) of sample aliquots (Schlee et al. 1995), so that a direct inter-study comparison was not feasible. However, they found an interesting trend for MDA with altitude. In particular, Schlee et al. (1995) found that MDA in *P. furfuracea* showed two peaks: a relative maximum at c. 1300 m and an absolute one at the timberline (c. 1900 m), perfectly matching the SOD activity pattern. Schlee et al. (1995) explained the contextual high enzymatic activity and MDA content as a species-specific adaptive response to oxidative stress caused by relatively high levels of tropospheric ozone possibly occurring at such elevations (Schlee, et al. 1995; Cristofanelli et al. 2007). Without point measures on ground ozone levels, it is not possible to exclude an effect of ozone altitudinal gradients on MDA response, also considered the highly performing anti-oxidant systems constitutive of the species, which may successfully determine the lowering of MDA content in case of decreasing ambient air ozone concentrations, not necessarily contradicting the general lowering of Chl$_a$F levels (Schlee 1995; Bertuzzi et al. 2013, 2018).

The variation pattern observed for the second fungal marker is also quite interesting. Ergosterol is the main sterol of fungal cells and is commonly used as biomarker of fungal vitality in lichens (Paoli et al. 2015). The concentration of ergosterol is mostly related with the basal respiration rates, but also with membrane integrity (Sundberg et al. 1999). The values measured in our test species (0.23 - 0.83 μg mg^{-1}) lied within the ranges reported for lichens in the literature (0.1 - 1.8 μg mg^{-1}; Dahlman et al. 2002; Bačkor and Loppi 2009). Ergosterol generally lowers in lichens in response to environmental stressors, such as metal loads (Tarhanen et al. 1999). Therefore, the drop of its values after the transplantation is consistent with enhanced stressing conditions experienced by the mycobiont (Vannini et al. 2016). Interestingly, at the source treeline sites, ergosterol contents in var. *ceratea* were substantially higher than those of the nominal variety, in line with the ecological

preferences of this taxon, generally more abundant at the imberline (Tretiach et al. 2013). Such pattern also matched the lowest MDA content revealed for var. *ceratea* at site A and B. Similarly, at the transplant sites A1-A4 and B1-B4, the specular patterns of ergosterol and MDA were fully consistent, indicating more stressing conditions for var. *ceratea*. It is worth to notice that MDA and ergosterol were both significantly correlated with RH (Spearman's rho of 0.49 and 0.46, respectively; $p < 0.05$), as appreciable by the comparable shapes of within-area trends of the microclimatic descriptor (Supplementary Fig. S3) and response variables (Fig. 3).

The influence of humidity on the distribution of the two varieties was previously highlighted at different spatial scales by correlational studies (e.g., in Great Britain and the Alps; Martellos 2003; Hawksworth and Chapman 1971). In particular, in the British Isles, the frequency of var. *ceratea* seemed to increase whith increasing humidity. However, significant deviations to this pattern were also found (e.g. Halvorsen and Bendiksen 1982; Tretiach et al. 2013). In Norway, a highly variable correlational pattern was found amongst the occurrence of olivetoric acid strains (var. *ceratea*), humidity and mean annual precipitation (Halvorsen and Bendiksen 1982). Also in the Alps the varieties may have similar frequencies in sites characterized by the same altitude and wet vs. drier mesoclimatic conditions (Tretiach et al. 2013). Besides the inconsistency of correlational observations, our preliminary results suggest that the relative humidity could have an effect on the intervarietal response of the species at the target spatial scale, as also possibly related to complex interplays with other environmental variables. As a matter of fact, at larger scales, other factors, including complex phenomena such as the founder effects, may have determined the occurrence of apparently contrasting distributional patterns.

4.2 Biotic factors: drivers or masking agents?

We highlighted the possible occurrence of biotic interactions with – still inscrutable – outcomes on the varietal distribution of *P. furfuracea*, consisting in its grazing by tree-climbing land slugs. Climbing gastropods are known to play a role in determining the lower distribution limit of epiphytic lichens along a vertical canopy gradient, influencing the spatial pattern of susceptible species (Asplund et al. 2010). Interestingly, the palatability for generalist gastropods (as *Lehmannia marginata* is; Rowson et al. 2014) depends on the species-specific lichen's chemical defence (Benesperi and Tretiach 2004; Gauslaa 2005), as proven for different Parmeliaceae by means of laboratory and field experiments (Černajová and Svoboda 2014). In our case, samples of either varieties were moderately grazed, although in sites B1 and B4 var. *ceratea* was apparently preferred, but this could have been due to the smaller size of exposed *ceratea* thalli.

It could be argued that intense grazing may affect the physiological assessment, however several precautions were taken in order to minimize such possibility. In particular, highly grazed thalli as well as the ones colonized by moulds were not used to assemble samples for MDA determination. Nonetheless there is no doubt that intense grazing may act as a confounding factor. On the other hand, the possibility that gastropod grazers may represent a biotic driver for the distribution of *P. furfuracea* varieties is an interesting hypothesis that would deserve to be tested.

Preliminary conclusions and future perspectives

In this work, the physiological response of *Pseudevernia furfuracea* varieties to environmental changes in a clean-air environment was evaluated by means of sample transplantation from higher to lower elevations. This is the first attempt to causally relate lichen physiology at the infraspecific levels to environmental drivers in a downscaled experimental frame.

At further confirmation that the identification of causality in complex systems is a difficult task (Sugihara et al. 2012), our preliminary analysis highlights a rather composite response of the varieties of *Pseudevernia furfuracea* to environmental changes, as caused by altitudinal shifts.
We found that the mycobiont markers better responded to the transplant, although not always consistently among each other and among areas characterized by different mesoclimatic conditions. Moreover, limited to the peroxidation of membrane lipids, the observed pattern was counterintuitive to a certain measure, determining the need to assess the altitudinal gradients of oxidant pollutants in the target areas.

Our preliminary findings also pave the way to assess the effects of canopy-related factors as well as those of gastropods grazing. For instance, further experiments might plan the sample exposure at different heights on trees, given the evidences that grazing can be avoided or substantially reduced at higher positions on trunks (Asplund et al. 2010).

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS S1

S1.1 Thin Layer Chromatography

Thin layer chromatography was preliminary performed on a randomly chosen set of 4 samples per collection site, one sample of the nominal variety (A*f*, B*f*, an C*f*) and three further of var. *ceratea* (c_1, c_2, c_3; Supplementary Fig. S1).

Extracts of the lichen material were obtained by soaking five terminal lobes per thallus in ~1ml of acetone for 10 minutes into 2 ml Eppendorf tubes. The concentrated solution was then used for spotting on the TLC plate using a capillary tube. After spotting, the plates were first observed under short wavelength ultraviolet light to assess the required spots intensity, following Elix and Ernst-Russell (1993). Afterwards, chromatographic development was performed using the common A (toluene: dioxane: acetic acid, 180:45:5), B (hexane: methyl tert-butyl ether: formic acid, 140:72:18) and C (toluene: acetic acid, 170:30) solvent system (Culberson 1972).

Acetone extracts of control specimens from the TSB herbarium, all known to contain atranorin (a very common lichen metabolite), and either olivetoric or physodic and oxyphysodic acids, were used as references in a previous chromatographic displacement of the tested lichen (Incerti et al. 2017; data not shown). Reference samples for var. *ceratea* were TSB40968 (*Pseudevernia furfuracea* var. *furfuracea* (L.) Zopf, with physodic and oxyphysodic acids), TSB40969 (*P. furfuracea* var. *ceratea* (Ach.) Hawksw., with olivetoric acid) and TSB15431 (*Cetrelia olivetorum* (Nyl.) Culb. & Culb., with olivetoric acid), whilst the reference for samples of the nominal variety was a purposedly collected specimen of *Hypogymnia tubulosa* (Schaer.) Hav., known to contain physodic and oxyphysodic acids (Orange et al. 2001). For this work, the previous identification of lichen substance patterns by TLC allowed their fast assessment directly using sampled *P. furfuracea* var. *furfuracea* as inner reference (Supplementary Fig. S1).

Silica gel pre-coated glass-backed plates of 20 × 20 cm (60G F254, Merck, Darmstadt) were used in the chromatography. In all solvent systems the spots on plates were placed 1 cm apart, 2 cm above the base, and the solvent was let travelling until 3 cm from the top of the plate to avoid edge effects. Once dried, plates were examined under visible light, and the positions of pigments appearing as coloured spots were recorded. Then, the plates were examined under short and long wavelength UV light (~254 nm and ~366 nm, respectively). UV-reactive spots were marked with a soft pencil and colours were recorded. After a brief drying, plates were treated with 10% sulphuric acid solution with a brush and then heated to 100 °C on a hotplate for 10 minutes in order to develop the spots: the colours after the charring were recorded.

S1.2 Climatic and microclimatic characterization of study sites

The climatic characterization of the study area was performed in a GIS environment using QGIS 2.18.9 ‘Las Palmas’ software, using climatic data from publicly available repositories (O’Donnel et al. 2012; www.worldclim.org). The following variables were considered, mapped at 30-arcseconds

spatial resolution (≈ 1 km) over the period 1960-1990: mean annual precipitation (MAP), precipitation of the wettest month (PWM), precipitation of the driest month (PDM), precipitation seasonality (PS), mean annual temperature (MAT), maximum temperature of the warmest month (TWM), minimum temperature of the coldest month (TCM), and temperature seasonality (TS). Mean values and associated standard deviations of climatic variables in the study sites were calculated by considering all the climatic data within circular buffers of 1.5 km radius centered in the study sites. A preliminary multivariate assessment and a correlation analysis (Sect. 2.4) led to the removal of temperature-related variables and highly correlated precipitation variables. For explanatory purposes, data are shown limited to MAP (Supplementary Fig. S2).

A microclimatic characterization was performed at each transplant site. Air temperature (T), relative humidity (RH) and dew point (i.e., DP: the temperature to which air become saturated with water vapor; Monteith and Unsworth 1990) were continuously monitored every hour by thermo-hygrometric sensors (EL-USB-1, Lascar Electronics, UK) installed at each site. Sensors, protected by plastic cover to avoid massive rain infiltration, were fixed on the same branches of lichen-hosting trees.

S1.3 Evaluation of the transplant effect

Autotransplants were carried out at the three collection sites (A, B and C) to test the effect of the exposure device on the physiology of lichen samples. The effect was assessed by measuring Chl$_a$F and MDA content, as described in Sects. 2.3.1 and 2.3.2.

At the moment of their sampling, at each site, c. 40 thalli thalli were sorted in the field according to their variety, and immediately mounted on four exposure devices (Sect. 2.2), two per variety. These were attached on larch branches and retrieved after 6 months (Fig. 1).

In this case, 18 F_v/F_m measurements were carried out, whereas MDA content was assessed on 5 samples.

In order to assess whether samples were substantially unaffected by the transplant itself, the median values of physiological markers in unexposed samples (i.e., before exposure) and after the 6-month exposure at the collection sites (i.e., autotranplants) were tested for significant differences using Mann-Whitney's U test for independent samples (Supplementary Table S1). Statistical significance was tested at $\alpha = 0.05$.

SUPPLEMENTARY RESULTS S1

S1.1 Effect of the lichen transplant

The results of the Mann-Whitney's U test for independent samples showed a pattern of limited physiological differences between unexposed samples and autotransplants. Indeed, concerning F_v/F_m values, these were significantly lower after the autotransplant in just one third of cases; i.e., in samples of var. *furfuracea* at site B and in samples of var. *ceratea* at site C (Supplementary Table S1). However, Chl$_a$F values after the autotransplant were consistent with fully healthy algal

populations, thus highlighting the absence of a detrimental effect on the photosynthetic apparatus potentially ascribable to the presence of the soft plastic net covering lichen thalli on the exposure devices.

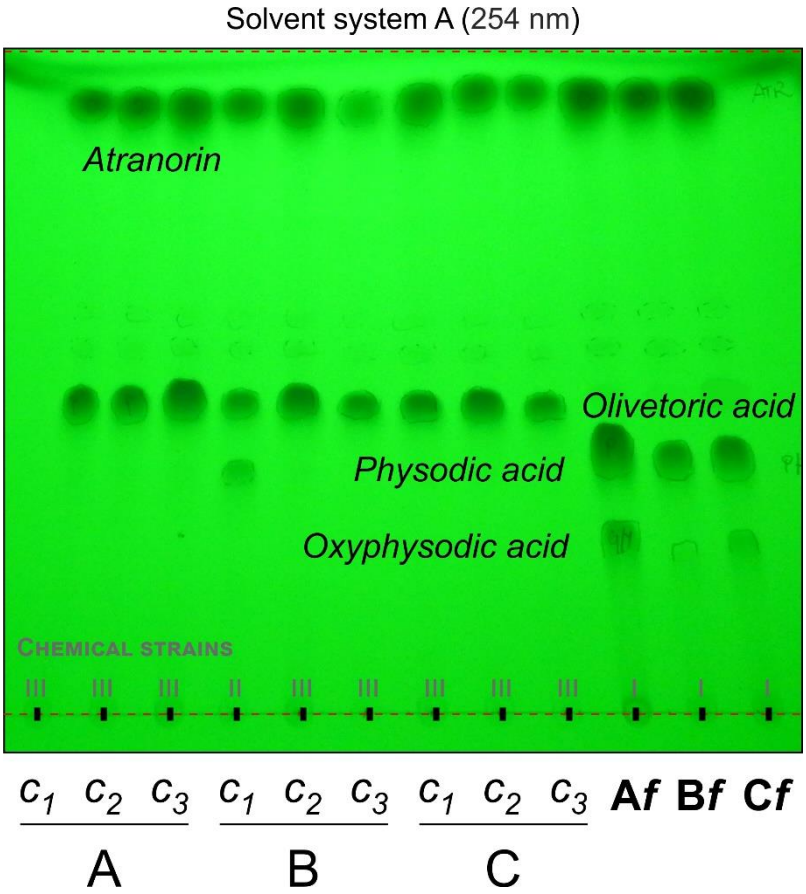
Moreover, MDA values exhibited significant differences only in the case of variety *ceratea* at site B. However, in this case, MDA content was higher before the exposure, thus furtherly excluding any adverse effect of the exposure devices.

Overall, such results suggested the neglectability of physiological effects strictly inherent to the experimental manipulation of samples, therefore allowing a proper evaluation of those caused by changes in environmental conditions.

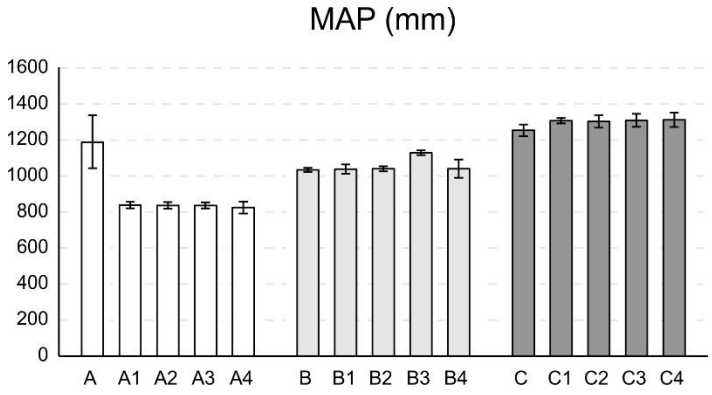
Supplementary Table S1. Mean vales and associated standard deviations of Chl$_a$F (F_v/F_m) and MDA values in unexposed samples ('before exposure') and samples transplanted for 6 months at the collection sites ('Autotransplant') (*f*: var. *furfuracea*; *c*: var. *ceratea*). The results of the Mann-Whitney test are also reported, with significant *p*-values ($p < 0.05$) highlighted in italic. Chl$_a$F assessment was carried out on 72 and 18 measurements, respectively for unexposed samples (Sect. 2.2) and autotransplants (Supplementary Methods S1.3); MDA content was assessed on 8 and 5 samples, respectively for unexposed samples (Sect. 2.2) and autotransplants (Supplementary Methods S1.3).

Collection site	Variety	Samples	F_v/F_m	Mann-Whitey test		MDA (nmol g^{-1})	Mann-Whitey test	
				U	*p*-value		U	*p*-value
A	*c*	Before exposure	0.722 ± 0.041	577.5	0.480	34.0 ± 4.6	13.0	0.341
		Autotransplant	0.733 ± 0.029			39.0 ± 8.0		
	f	Before exposure	0.741 ± 0.030	464.0	0.064	44.0 ± 12.7	15.0	0.510
		Autotransplant	0.757 ± 0.053			51.5 ± 14.8		
B	*c*	Before exposure	0.739 ± 0.036	476.5	0.085	46.7 ± 9.3	3.0	*0.016*
		Autotransplant	0.756 ± 0.020			31.1 ± 3.0		
	f	Before exposure	0.728 ± 0.037	384.0	*0.008*	55.1 ± 8.3	14.0	0.421
		Autotransplant	0.691 ± 0.054			57.2 ± 17.5		
C	*c*	Before exposure	0.750 ± 0.021	378.5	*0.007*	41.1 ± 7.2	15.0	0.510
		Autotransplant	0.725 ± 0.039			44.5 ± 8.4		
	f	Before exposure	0.734 ± 0.030	457.0	0.055	31.9 ± 5.2	19.0	0.942
		Autotransplant	0.710 ± 0.063			32.7 ± 7.7		

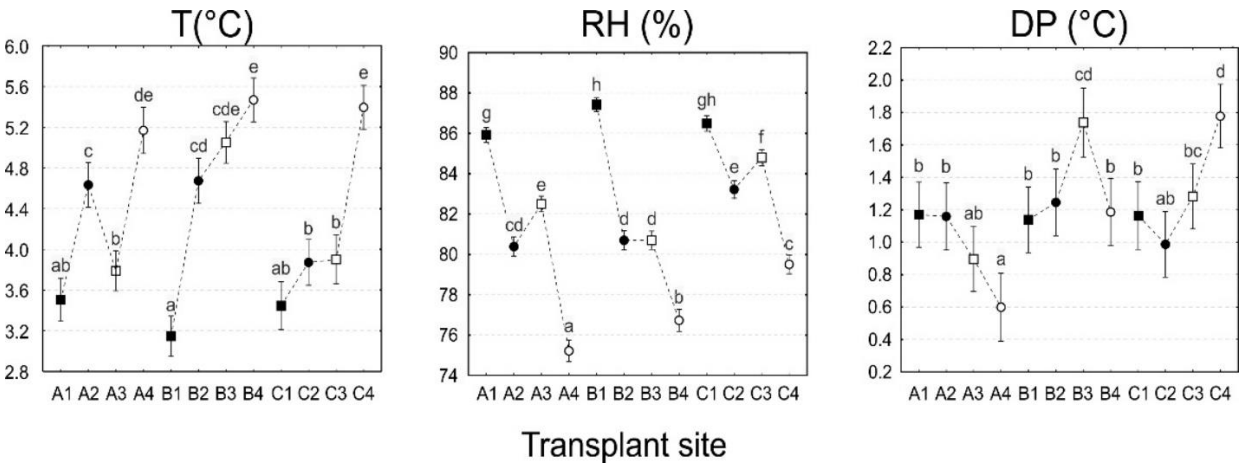
SUPPLEMENTARY FIGURES S1-S4



Supplementary Figure S1. TLC plate (solvent A) under 254 nm wavelenght. Spots corresponding to atranorin, physodic, oxyphysodic and olivetoric acids are visible, and chemical strains are reported above the spots of sample extracts (dotted base line). Capital letters (A, B, C) indicate the study area; c_1-c_3 are three samples of var. *ceratea*, whereas A*f*, B*f* and C*f* are reference samples of the nominal variety.



Supplementary Figure S2. Mean annaul precipitation anda ssociated standard deviations (MAP) at the study sites (A, B, C: treeline collection sites; A1-A4, B1-B4, C1-C4: transplant sites at c. 1000 m a.s.l.).



Supplementary Figure S3. Temperature (T), relative humidity (RH) and dew point (DP) as measured at the transplant sites. Data refer to mean values and 95% confidence intervals. Different letters indicate significant differences (Dunn's post hoc test at $p < 0.05$).

Supplementary Figure S4. *Lehmannia marginata* land slug grazing *Pseudevernia furfuracea* var. *furfuracea* (a) and var. *ceratea* (b) thali. *L. marginata* sheltering inside the lichen exposure device (c).

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APPENDIX II

SCIENTIFIC PRODUCTION

RESEARCH PAPERS IN INTERNATIONAL JOURNALS

Cecconi E, Fortuna L, Pellegrini E, Lorenzini G, Nali C, Tretiach M. (2019) Beyond ozone-tolerance: Effects of ozone fumigation on trace element and PAH enriched thalli of the lichen biomonitor *Pseudevernia furfuracea*. *Atmospheric Environment* 210, 132-142.

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 (2019) New interpretative scales for lichen bioaccumulation data: the Italian proposal, *Atmosphere* 10, 136-154.

Cecconi E, Incerti G, Capozzi F, Adamo P, Bargagli R, Benesperi R, Candotto Carniel F, Cristofolini F, Favero Longo SE, Giordano S, Puntillo D, Ravera S, Spagnuolo V, Tretiach M (2019) Background element content in the lichen *Pseudevernia furfuracea*: A comparative analysis of digestion methods. *Environmental Monitoring and Assessment* 191, 260.

Cecconi E, Incerti G, Capozzi F, Adamo P, Bargagli R, Benesperi R, Candotto Carniel F, Cristofolini 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.

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) Infraspecific 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(9), 8004-8016.

MANUSCRIPTS SUBMITTED TO INTERNATIONAL JOURNALS

Cecconi E, Fortuna L, Peplis M, Tretiach M. Element accumulation performance of living and dead lichens in a large sample-sized transplant application [Submitted to *Ecological Indicators*; February 18th 2020, ECOLIND-16023].

ABSTRACTS IN NATIONAL JOURNALS

Cecconi E (2019) Monitoring environmental changes by lichens: Methodological aspects and applications. *Notiziario della Società Lichenologica Italiana* 32, 133-137 [*oral presentation*].

Fortuna L, **Cecconi E**, Peplis M, Tretiach M (2019) Biomonitoraggio delle emissioni associate alla co-combustione di idrocarburi fossili e combustibile solido secondario (CSS) mediante trapianti lichenici: Un caso di studio nell'alto Pordenonese. *Notiziario della Società Lichenologica Italiana* 32, 52 [*poster*].

Cecconi E, Fortuna L, Peplis M, Tretiach M (2019) Confronto tra la capacità di accumulo di elementi in talli vivi e devitalizzati di *Pseudevernia furfuracea*: *When 'dead' is not so bad*. *Notiziario della Società Lichenologica Italiana* 32, 34 [*oral presentation*].

Cecconi E, Pellegrini E, Fortuna L, Bertuzzi S, Lorenzini G, Nali C, Tretiach M (2018) Beyond O_3-tolerance. Sviluppi sulla valutazione degli effetti combinati di inquinanti sulla fisiologia di un illustre biomonitor. *Notiziario della Società Lichenologica Italiana* 31, 25 [*oral presentation*].

Cecconi E, Incerti G, Capozzi F, Adamo P, Bargagli R, Benesperi R, Candotto Carniel F, Cristofolini F, Favero Longo SE, Giordano S, Puntillo D, Ravera S, Spagnuolo V, Tretiach M (2017) Variabilità metodologica in studi di bioaccumulo e caratterizzazione del contenuto elementare di background in *Pseudevernia furfuracea*. *Notiziario della Società Lichenologica Italiana* 30, 29 [*oral presentation*].

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ABSTRACTS IN CONFERENCE PROCEEDINGS

Cecconi E, Ongaro S, Micai G, Ait Kaci M, Tretiach M (2019) Infraspecific physiological response of the macrolichen *Pseudevernia furfuracea* to environmental changes: An altitudinal transplant experiment in a clean-air region of the Alps. *Joint congress of the Italian Society of Plant Biology and the Italian Botanical Society (SIBV/SBI)*, Padova, 4-6 September 2019 [*poster*].

Cecconi E, Incerti G, Capozzi F, Adamo P, Bargagli R, Benesperi R, Candotto Carniel F, Cristofolini F, Favero Longo SE, Giordano S, Puntillo D, Ravera S, Spagnuolo V, Tretiach M (2018) Background element content of the lichen *Pseudevernia furfuracea*: A comprehensive overview. From the supranational state of art to a new methodological framework for the assessment of regional benchmarks. *Proceedings of the 8th International Workshop on Biomonitoring of Atmospheric Pollution* (BIOMAP8) 8, 21 [*oral presentation*].

OTHER PRODUCTS

Giordani P, Benesperi R, Bianchi E, Brunialti G, **Cecconi E**, Contardo T, Di Nuzzo L, Fortuna L, Frati L, Loppi S, Monaci F, Munzi S, Nascimbene J, Paoli L, Ravera S, Tretiach M, Vannini A. Italian Guidelines for the Use of Lichens as Bioaccumulators [*ready to be published online by the Italian Institute for Environmental Protection and Research, ISPRA*].

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ARTICLES IN PREPARATION

Cecconi E, Fortuna L, Tretiach M. How many samples? Costs and background data variability in the lichen transplant technique: A trade-off. [*Article in prep.*].

Fortuna L, **Cecconi E**, Tretiach M. *Ante-post operam* lichen biomonitoring of element and PAH depositions around a cement plant subjected to combustion system transformation [*Article in prep.*].

AWARDS

International "Gaggi Award" for the best Ph.D. thesis offered by the Italian Lichen Society (2019). http://www.lichenologia.eu/index.php?procedure=bandi_gaggi.