Development of a toolbox to biomonitor air quality: towards the sustainability of the Azorean green environment

Tese de Doutoramento

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Under the scientific supervision of:

- Professora Doutora Patrícia Ventura Garcia
- Professor Doutor Armindo dos Santos Rodrigues
- Professora Doutora Cristina Maria Branquinho Fernandes

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A dissertation submitted to the University of the Azores in fulfillment of the requirements for the degree of Doctor of Philosophy in Biology (Biodiversity branch), under the scientific supervision of Professora Doutora Patrícia Ventura Garcia, assistant professor of the Faculty of Sciences and Technology of the University of the Azores, Professor Doutor Armindo dos Santos Rodrigues, associate professor with habilitation of the Faculty of Sciences and Technology of the University of the Azores, and Professora Doutora Cristina Maria Branquinho Fernandes, associate professor with habilitation of the Faculty of Sciences of the University of Lisbon.

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Statement of Authorship

I hereby testify that this thesis was made of my own initiative and especially developed to this purpose. All scientific data obtained throughout this PhD project and herein presented was submitted, peer-reviewed and published in indexed scientific journals as the following research articles:

Bernardo, F., Rodrigues, A., Branquinho, C., Garcia, P., 2020. Elemental profile of native lichens displaying the impact by agricultural and artificial land uses in the Atlantic island of São Miguel (Azores). *Chemosphere* 267 (March 2021), 128887. https://doi.org/10.1016/j.chemosphere.2020.128887 (Q1; IF2019 = 5.778).

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Bernardo, F., Rocha, T., Branquinho, C., Garcia, P., Rodrigues, A., 2020. Thallus structural alterations in green-algal lichens as indicators of elevated CO_2 in a degassing volcanic area. *Ecological Indicators* 114, 106326. https://doi.org/10.1016/j.ecolind.2020.106326 (Q1; IF2019 = 4.229).

As these studies were carried out in collaboration with other authors, I also declare that I was the main responsible for designing and carrying out the experimental and field work, as well as interpreting the results, composing the manuscripts and submitting the research papers for publication as the first author.

Filipe Miguel Teixeira de Sousa Bernardo

(The validated digital signature is at the last page of this thesis)

Dedicado à minha mãe

O que seria de mim sem o teu sacrifício, abnegação e imensa paciência?

«A mother is she who can take the place of all others but whose place no one else can take.»

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Abstract

The legal regime concerning air quality and protection of the atmosphere in the Azores upholds the necessity of striving towards harmonizing human socioeconomic development with the preservation of excellent standards of air quality. Though historically a relatively incipient issue, contemporary challenges posed by intensifying anthropogenic activities or climate change require further efforts to address and prevent the decline of air quality, through increasing emissions of atmospheric pollutants, entailing the degradation of the natural patrimony of the Azores and adverse effects upon public health. Furthermore, owing to archipelago′s volcanic background, volcanogenic emissions continually occur during periods of quiescent activity, particularly affecting the populations chronically exposed in the proximity of active volcanoes. Hence, using lichens as biomonitors, the general aim of this thesis was to refine a set of tools to biomonitor air pollution in selected environmental scenarios of São Miguel island (SMG), addressing both anthropogenic and volcanogenic airborne emissions originating, respectively, from the unprecedented intensification of human activities as well as ongoing manifestations of active volcanism.

The broad-spectrum elemental profile of naturally occurring (native) foliose *Parmotrema* lichens, sampled across the land use gradient of SMG, revealed a transversal pattern of significant elemental enrichment at agricultural and even more at artificial areas compared with the more pristine-like background of forest areas, thus tracing environmental contamination, through airborne emissions, displaying the impact of intensifying agricultural and urban activities, particularly in the context of the greater environmental vulnerability inherent to small oceanic island ecosystems. Some potentially toxic heavy metals (Pb, Mo, Mn, V, Zn, Th, Sb, Sn, Co, Ni, Cu, Cr, Ti, Fe) reached high bioaccumulation levels in lichens from artificial land use, while nitrogen bioaccumulation was highest in those from agricultural land use, reflecting the ongoing N pollution that has caused eutrophication issues in recent decades at SMG. The N isotopic signature ($\delta^{15}N$) was a suitable tracer to distinguish the nitrogenous contribution between agricultural and artificial sources, with applicability to discern its respective origin in more complex scenarios of spatial overlapping of these sources.

Meanwhile, an alternative approach was followed to biomonitor volcanic air pollution by transplanting fruticose *Usnea rubicunda* lichens from a reference site into several sites at Furnas village, inside the caldera of the actively degassing Furnas volcano. After a prolonged exposure period of 6 months, it was verified that the $\delta^{34}S$ isotopic ratio, retained in the transplanted lichens, is a specific tracer of airborne hydrothermal emissions in outdoor conditions, strongly relating with the distance to fumarolic fields on a logarithmic regression. $\delta^{34}S$ data was subsequently combined with spatial data on habitational areas to develop a method for spatially modelling, with high spatial resolution and time integration, the risk

areas of chronic exposure to air pollution in volcanic environments. Moreover, as revealed through an histomorphometric methodology, the thalli of these same transplanted lichens displayed significant structural alterations suggesting a positive and time-integrated response of greater efficiency, by the algal partner, in sustaining the production of additional fungal biomass. These structural alterations in the heteromerous thalli of *U. rubicunda* are sensitive responses reflecting the extended exposure under greater availability of CO_2 in ambient conditions, with potential to indicate a global rise of CO_2 levels.

The outputs of this thesis demonstrate the expediency of complementing conventional air quality monitoring routines in the Azores, currently limited to three (rural and urban) background monitoring stations, with biomonitoring tools under more focused environmental assessments, provided its cost-effectiveness, augmented environmental representativity, greater spatial distribution as well as meaningful extrapolation of data on atmospheric pollutants in terms of time-mediated effects upon the biosphere and public health. Considering the susceptibility of small oceanic islands to disrupting factors, these advantages are valuable for developing further strategies towards preserving air quality in face of the threats posed by volcanic background of the archipelago and ongoing intensification of human activities, mainly driven by livestock, tourism, and urbanization.

Keywords: Air quality; biomonitoring; lichens; island ecosystems; volcanic emissions.

Resumo

O regime jurídico da qualidade do ar e proteção da atmosfera dos Açores prevê a necessidade de encontrar soluções no sentido de harmonizar o desenvolvimento socioeconómico humano com a preservação dos padrões de excelência da qualidade do ar. Embora historicamente tenha sido um problema incipiente, os desafios contemporâneos colocados pela intensificação das atividades antropogénicas ou alterações climáticas requerem esforços adicionais dedicados à prevenção da declínio da qualidade do ar, por via do aumento das emissões de poluentes atmosféricos, conduzindo à degradação do património natural dos Açores e efeitos adversos sobre a saúde pública. Além disso, devido à origem vulcânica do arquipélago, emissões vulcanogénicas decorrem continuamente durante períodos de atividade quiescente, afetando particularmente as populações cronicamente expostas na proximidade de vulcões ativos. Assim, através do uso de líquenes como biomonitores, o objetivo geral desta tese foi afinar um conjunto de ferramentas para biomonitorizar a poluição do ar em cenários ambientais característicos da ilha de São Miguel (SMG), abrangendo tanto as emissões atmosféricas antropogénicas como vulcanogénicas originadas, respetivamente, pela intensificação sem precedentes das atividades humanas e pelas incessantes manifestações de vulcanismo ativo.

O perfil elemental de largo espectro de líquenes foliosos nativos (de ocorrência natural) do género *Parmotrema*, amostrados ao longo do gradiente de uso do solo de SMG, revelou um padrão transversal de enriquecimento elemental em áreas agrícolas e sobretudo áreas artificiais em comparação com o fundo pristino de áreas florestais, refletindo a contaminação ambiental causada por emissões atmosféricas e exibindo o impacto da intensificação das atividades agrícolas e urbanas, particularmente no contexto da acrescida vulnerabilidade ambiental inerente aos ecossistemas de pequenas ilhas oceânicas. Alguns metais pesados potencialmente tóxicos (Pb, Mo, Mn, V, Zn, Th, Sb, Sn, Co, Ni, Cu, Cr, Ti, Fe) atingiram níveis de bioacumulação elevados nos líquenes do uso do solo artificial, enquanto a bioacumulação de azoto foi mais elevada nos líquenes do uso do solo agrícola, indicando a poluição azotada que tem causado problemas de eutrofização nas últimas décadas em SMG. A assinatura isotópica $\delta^{15}N$ revelou-se um indicador adequado para distinguir a contribuição azotada entre fontes agrícolas e artificiais, com aplicabilidade para o rastreamento da respetiva origem em cenários mais complexos de sobreposição espacial destas fontes.

Entretanto, foi escolhida uma abordagem alternativa para biomonitorizar a poluição vulcânica do ar através do transplante de líquenes fruticulosos, da espécie *Usnea rubicunda*, recolhidos a partir de um local de referência e transplantados para vários locais na vila das Furnas, localizada dentro da caldeira do vulcão que se encontra em desgaseificação ativa. Depois de um período de exposição prolongado de 6 meses, verificou-se que a razão isotópica

de enxofre ($\delta^{34}S$), retida nos líquenes transplantados, é um indicador específico de emissões hidrotermais em condições ao ar livre, relacionando-se fortemente com a distância aos campos fumarólicos numa regressão logarítmica. Os dados de $\delta^{34}S$ foram posteriormente combinados com dados espaciais das áreas habitacionais para desenvolver um método de modelação espacial das áreas de risco de exposição crónica a poluição do ar em ambientes vulcânicos, com elevada resolução espacial e integração temporal. Além disso, tal como revelado através de uma metodologia histomorfométrica, os talos destes mesmos líquenes transplantados exibiram alterações estruturais significativas que sugerem uma resposta positiva de maior eficiência ao longo do tempo, pelo parceiro algal, na produção de biomassa fúngica adicional. Estas alterações estruturais no talo heterómero da *U. rubicunda* são respostas sensíveis que refletem a exposição prolongada sob maior disponibilidade de CO_2 no ar ambiente, com potencial para indicar um aumento global dos níveis de CO_2.

Os resultados desta tese demonstram a pertinência de complementar as rotinas de monitorização convencional da qualidade do ar nos Açores, atualmente limitadas a três estações de monitorização (rural e urbana) de fundo, com ferramentas de biomonitorização enquadradas em estudos ambientais mais focados, dada a sua rentabilidade, maior representatividade ambiental e distribuição espacial, permitindo também traduzir o significado dos dados de poluentes atmosféricos em termos de efeitos ao longo do tempo sobre a biosfera e saúde pública. Considerando a suscetibilidade particular de pequenas ilhas oceânicas a fatores disruptivos, estas vantagens são valiosas para o desenvolvimento de estratégias de preservação da qualidade do ar, atendendo às ameaças colocadas pela natureza vulcânica do arquipélago e pela corrente intensificação das atividades humanas, propulsionadas pela agropecuária, turismo e urbanização.

Palavras-chave: Qualidade do ar; biomonitorização; líquenes; ecossistemas insulares; emissões vulcânicas.

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CHAPTER 1

General introduction

1.1. Chronic exposure to air pollution in volcanic environments

Volcanic activity is one of the most relevant and ceaseless natural sources of air pollution, posing threatening implications for the health of nearby ecosystems and human populations (Rodrigues and Garcia, 2015; Heaviside et al., 2021) Human settlement at volcanic areas is majorly by their nutrient-rich fertile soils, even despite potentially disastrous seismic and eruptive events. Recently updated assessments of the worldwide distribution of the population between 1975 and 2015 in relation to recent eruptive volcanism estimate that more than 1 billion people live within 100 km of a Holocene volcano, with higher a concentration in the range between 10 and 20 km (Freire et al., 2019).

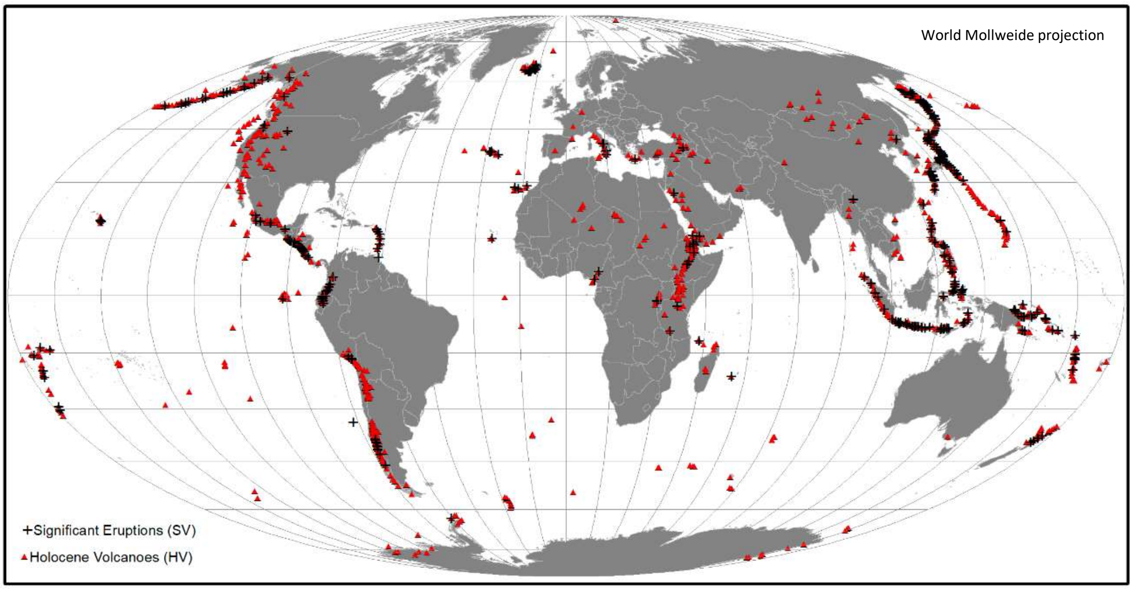

Figure 1. Distribution of two global volcano datasets: Holocene Volcano List v4.7.6 and NOAA Significant Volcanic Eruption Database. Map published by Freire et al. (2019).

With population density increasing around volcanoes, it is unsurprising that relatively less concern is given to less apparent and less abrupt hazards related to volcanic activity, such as chronic exposure to toxic gases and particulate matter (PM) continually released during the longer inter-eruptive periods (Horwell et al., 2015; Amaral and Rodrigues, 2019). Once the eruptive overflowing discharge subsides, volatile volcanogenic gases and particles, emanating from the main crater, fumarole fields or through soil diffuse degassing become the most hazardous emissions to consider during quiescent stages, including the gases CO and CO_2, SO_2, HCl, HF, H_2S and radon, as well as potentially harmful metals and metalloids such as Al, As, Cd, Cu, Hg, Rb, Pb and Zn (Hansell et al., 2006; Rodrigues and Garcia, 2015; Amaral and Rodrigues, 2019). These pollutants can cause adverse health effects even at low but constant concentrations given the chronic exposure (possibly lasting an entire lifetime) to their increased release and bioavailability under volcanic environments (Table 1).

Table 1: Summary of the main potential adverse health effects of long-term exposure to volcanic pollutants (Rodrigues and Garcia, 2015; Amaral and Rodrigues, 2019).

Pollutant	Potential adverse health effects of chronic exposure
CO	Increased risk of heart disease; post-poisoning sequelae
CO_2	Lethargy; recurring headaches; dyspnea; brain developmental impairment
SO_2	Eyes and skin irritation; asthma exacerbation; chronic bronchitis
HCl	Skin irritation; inflammation, hyperplasia, and ulceration of mucosal membranes and respiratory tract; lung injury; teeth discoloration and erosion
HF	Dental fluorosis; skeletal fluorosis; kidney and liver lesions
H_2S	Nervous and cardiovascular disorders; COPD; pulmonary edema
Rn	Lung cancer
As	Alterations in skin pigmentation; keratosis
Cd	Osteoporosis; kidney damage; carcinogenicity
Hg, Pb	Neurotoxicity
PM (ash)	Silicosis; COPD; aggravation of asthma, bronchitis, or emphysema; chronic conjunctivitis; skin irritation; pleural mesothelioma

Moreover, as the need for identifying and mitigating risks associated with non-eruptive volcanism is being increasingly acknowledged by researchers and policy makers, still further research is needed to fill the gaps at multiple levels, such as: accurately predicting the duration and pathways of exposure given the temporal and spatial variability of airborne pollutants; measuring synergistic effects of complex low-level pollutant mixtures even if each component is within presumably safe levels; specifically associating exposure to health outcomes; evaluating the effectiveness of mitigation measures in reducing the exposure and improving the population health status (Rodrigues and Garcia, 2015; Amaral and Rodrigues, 2019). On each of these levels, new or improved monitoring and biomonitoring tools are valuable for applying measures meant to reduce the vulnerability of the populations.

However, the significance of chronic exposure to manifestations of active volcanism is not limited to the effects upon people. Given that volcanic areas can be of the most extreme environments on planet Earth, biomonitoring with an ecological perspective anticipates many more interesting research topics (Lattanzi et al., 2020). How do organisms and communities of diverse complexity respond to the extreme conditions found on these environments? Which mechanisms do they develop, at the various levels of biological organization, to cope with the hostility posed by high temperatures, acidic medias, constant degassing, or exceeding concentrations of pollutants? Volcanoes provide a natural setting where organisms face the co-occurrence of these multiple stressors under *in-situ* conditions, which is hard to replicate in laboratory. The implications of the resulting findings might lead to significant advances in understanding the processes that govern life, to insight on drivers of environmental change or to inspire novel biotechnological and biomedical applications.

1.2. The contribution of biomonitoring to environmental surveillance

A fitting analogy leading to an elementary definition of biomonitoring has been given by Friberg et al. (2011), that is, the use of biota to gauge and track environmental changes in their terrestrial or aquatic systems, much like the observation of symptoms to identify and follow up disease in medicine. These "symptoms" correspond to measurable biological responses, spanning the entire range of biological organization, from the gene to the ecosystem, providing means of interpreting environmental levels of pollutants in biological terms and assessing how they affect the health and wellbeing of living systems (Hershey et al., 2010; Rodrigues and Garcia, 2015; Walker et al., 2016). Organisms displaying these responses are conventionally called biomonitors, if referring to a quantitative determination of pollutants or others stressors, or ecological indicators if referring to assessing ecosystem diversity, structure or processes to indicate its condition (Holt, 2010; Branquinho et al., 2015). Biomonitoring approaches include (Hershey et al., 2010; Walker et al., 2016):

a) molecular and cellular measures of biochemical and physiological processes;

b) effects at the organism level, such as changes in morphological and behavioral traits or life history parameters, and relating them to measures of the bioaccumulated concentrations of pollutants in the organism and other biotic and abiotic measures;

c) population-level or community-level responses, such as the variations in population density or variations in patterns of species abundances displaying differing degrees of sensitivity/tolerance to the influence of a discriminating environmental stressor;

d) ecosystem-scale effects of stressors on ecological function, services, and processes.

Therefore, the key advantage of biomonitoring is to substantiate physical and chemical data from the environment by biologically meaningful data from living material, a crucial interpretation to understand causalities between environmental factors and effects on the biosphere (Garty, 2001; Walker et al., 2016). For instance, air pollution refers to the presence and quantity of pollutants in the atmosphere, while air quality refers to the burden of such pollution over living organisms interacting with it (Garty, 2001). Such burden cannot be accurately determined with measurements of physical and chemical parameters in the abiotic environment (instrumental monitoring of the atmosphere, henceforth termed conventional monitoring) and knowledge of the potential toxicity of hazardous pollutants, as the effects on living organisms are further conditioned by (Walker et al., 2016):

a) the spatial and temporal variability of the concentrations of pollutants in the air;

b) the degree of bioavailability for incorporation from the air into living organisms;

c) the degree of exposure of the organisms to the bioavailable fraction;

d) the diverse susceptibility between organisms to be adversely affected as well as their ability to cope with and tolerate the assimilated fraction.

Numerous factors influence the bioavailability of pollutants, such as temperature, pH, rainfall or interaction with other substances, making it very difficult to accurately predict the extent to which they are assimilated by organisms and their ensuing biological effects that are expressed in complex realistic field conditions rather than standardized conditions of laboratory experiments (Walker et al., 2016). Thus, conventional monitoring only gives an indirect measure of the degree of exposure, not providing information on the loads of pollutants inside the organisms and, even less, on the adverse effects that are caused by them (Rodrigues and Garcia, 2015).

Without biomonitoring assessments of the environment, it is not only problematic to predict the reaction of organisms, populations and communities to the complex interaction of biotic and abiotic factors, but also unattainable to determine the full spectrum of pollutants in circulation, their metabolites and co-metabolism products, due to analytical and economical limitations (Bondaruk et al., 2015). Contaminants can occur in exceedingly low concentrations, whose detection requires tedious and sensitive analytical methods, at prohibitive costs to ensure decent replication and spatial coverage, and often reflecting instantaneous transient conditions at the moment of sampling (Hershey et al., 2010; Holt, 2010). In contrast, biomonitors provide information on multiple pollutants in the same matrix with temporal integration corresponding to their life span or residence time in a particular system, reflecting long-term trends (Holt, 2010). For instance, metal pollutants accumulate in biological tissues (bioaccumulation), amplifying along the food chain (biomagnification), and can reach biologically meaningful levels over time, even if occurring at trace amounts (Holt, 2010; Bondaruk et al., 2015). Furthermore, since biomonitors are already populating the environment, they are easier and cheaper to sample recurringly, usually enabling both higher spatial representativeness of sampling sites than monitoring facilities and higher sampling frequency than instrumental field surveys (Augusto et al., 2004). Lastly, unlike conventional monitoring, biomonitoring takes into consideration the effects of the more unpredictable synergistic or antagonistic interactions of pollutant mixtures (Bondaruk et al., 2015; Rodrigues and Garcia, 2015) and can disclose indirect associations between chemical contamination and biotic effects (Holt, 2010).

As such, by definition, without biomonitoring, it is not possible to accurately assess and control air quality, regardless of the amount of data on air pollution. Hence, the tendency of current environmental monitoring programs has been to integrate biomonitoring methods providing complementary informative capability regarding the multitude of environmental stressors that influence environmental health (Bondaruk et al., 2015; Rodrigues and Garcia, 2015). A successful biomonitoring approach is determined by the choice of a suitable organism characterized by several hallmark traits (Holt, 2010; Walker et al., 2016).

Table 2 : Main traits of a suitable ecological indicator (Holt, 2010; Walker et al., 2016).

Indicator ability	► Robust to environmental variability, remaining relatively stable in an area despite human presence and moderate climatic variation. ► Sensitive to the disturbance or stressor at very low levels and very high levels without experiencing mortality (except in community-level studies). ► Responsive by providing measurable responses proportionally to the degree of contamination or alteration. ► Reflects the tendency of the population/community/ecosystem it is inserted in. ► Reliable and reproducible by displaying similar responses to the same exposure and the same factor in different studies in different locations.
Relevance	► Common with wide global distribution. ► Easily found and collected to facilitate reproducibility comparisons. ► Occurring abundantly with adequate local population density within the study area (avoid endangered and rare species). ► Economical value or societal significance, perhaps already harvested for other purposes, raising public interest and awareness regarding the problem being studied. ► Plays an important role in the ecosystem functions and services, so that the observed effects are ecologically meaningful.
Established	► Ecology, life history and life cycles well understood. ► Taxonomically stable and well documented. ► If possible, already proven a successful ecological indicator in past studies. ► Easier and cheaper to collect/cultivate/raise, survey and analyze.

Although biomonitoring is not without its drawbacks, which should be as minimized as possible in every experimental design, such as the overlap of natural variability and natural events (predation, parasitism, disease) with the influence of a given disturbing factor, or the potential oversimplification in extrapolating conclusions drawn from a single species to other species or even whole heterogeneous ecosystems (Holt, 2010), pairing biomonitoring approaches with conventional monitoring is highly commendable to surveil the action of environmental stressors upon natural systems (Friberg et al., 2011). By improving the understanding of the relationships between abiotic and biological components with temporal integration and spatial representativity, properly used ecological indicators tend to be better sensors of the status of environmental health and suitable to evaluate the impact of human activities on ecosystems (Branquinho et al., 2015), providing an integrated and cumulative outlook combining multiscale and multifactorial information (Hershey et al., 2010) that is crucial to anticipate environmental degradation before it is too late to reverse it.

1.3. Lichens as outstanding biomonitors of air pollution

Lichens are amongst the most peculiar and intriguing symbiotic organisms found on Earth, owing to the complex and ambiguous nature of the relationship between the fungal heterotrophic partner (mycobiont) housing the photosynthetic algae or cyanobacteria partners (photobiont) (Nash, 2008). Over the years, this relationship has been regarded from controlled parasitism by the fungi over the photobiont to a classic case of mutualistic association benefitting all partners, with a wide-ranging degree of lichenization observed between lichens. Either way, lichenization is a notable example of successful symbiosis, allowing the colonization of almost all terrestrial habitats, reaching beyond where the bionts separately are unable to thrive (Ahmadjian, 1993; Nash, 2008).

Besides their dual nature, lichens are generally characterized by other distinctive biological traits, despite their vast diversity in size, color, and thallus morphological structure. Most lichens are perennial and long-living, conserving a uniform morphology over time, usually growing relatively slowly, and occurring commonly as epiphytes on trees and other plants as well as colonizing the soil and rocks (Nash, 2008). Lichens are heavily dependent on water availability for survival as they lack specialized structures to regulate water and gas exchange and thus their water content is passively determined by surrounding environmental levels (poikilohydry). Consequently, the thallus surface is easily permeable to absorption of gases with water and dissolved substances while also enhancing trapping of particles from which they derive mineral nutrients. Moreover, lichens are devoid of roots and vascular system, incorporating relatively little content from the underlying substrate. Their largely passive and indiscriminate deep reliance on atmospheric dry and wet deposition means the chemical composition of lichens directly reflects the availability of elements through airborne emissions in the environment, making them one of the most reliable biomonitors of air pollutants (Conti and Cecchetti, 2001; Garty, 2001; Bargagli and Mikhailova, 2002; Batts et al., 2004; Nash, 2008; Bačkor and Loppi, 2009; Van der Wat and Forbes, 2015). Since most vascular plants are affected by root soil uptake, and other non-parasitic epiphytes (bromeliads, orchids, ferns) are still able to regulate their atmospheric uptake through cuticle and stomata, their dependence on atmospheric deposition, conjugated with their inability to regulate water and elemental exchange, makes lichens exceptionally suited organisms for the evaluation of the effects of atmospheric environmental changes, as the influence of soil and/or water is discriminated from the influence of the air (Augusto et al., 2015; Branquinho et al., 2015).

Considering their slow growth, longevity, sessile state, conservative structure, without shedding or renewing parts during the life cycle, and nutrient concentration mechanisms, more resistant lichens continuously bioaccumulate mineral elements above their physiological needs in their thalli, integrating the deposition of airborne elements from their surroundings over long periods of time (Garty, 2001; Bargagli and Mikhailova, 2002; Nash, 2008). This long-term integration capability has been used to reconstruct the spatial and temporal deposition patterns of air pollutants, most notably for those occurring in low amounts, as they keep concentrating to readily detectable levels in the lichen thalli. Such data is of critical significance in subsequent human health studies, considering the challenging aim of associating the chronic exposure to low-level hazardous air pollutants with their potential long-term effects in the population (Bargagli and Mikhailova, 2002; Augusto et al., 2007; Ribeiro et al., 2010; Van der Wat and Forbes, 2015).

Obtaining detectable concentrations of low-level air pollutants through instrumental measurements is typically less advantageous, requiring large volumes of air sampling for long periods (Van der Wat and Forbes, 2015). Moreover, instrumental measurements directly in the air provide a highly variable, short-term indication of airborne pollution with limited spatial and temporal extrapolation. The data based on conventional air quality monitoring usually consists of series of snapshots of instantaneous concentrations values of a limited number of pollutants with the sparse spatial representativity presented by a few stations. Hence, over the recent decades, the long-term temporal integration, particularly of low-level pollutants, and the high sampling density allowed by lichens are two key factors validating the use of lichen biomonitoring methods to counter the constraints of conventional monitoring (Augusto et al., 2007, 2009; Ribeiro et al., 2010).

Although the elemental concentrations in lichen thalli mainly correspond to the loads of incident airborne pollutants, short-term events such as rainfall, besides lichen physiological properties and turnover mechanisms, might induce temporal variations in thallus elemental content (Branquinho et al., 2008; Bačkor and Loppi, 2009). Furthermore, where multiple pollution sources co-exist, which is likely at larger spatial scales, it is harder to track the sources of pollutants and determine their relative contribution (source-apportionment) (Pinho et al., 2017). In these more complex settings, the analysis of the stable isotopic composition of lichens allows the tracing of the sources of atmospheric elements, since elemental forms emitted from different sources carry a distinct isotopic signal (Felix and Elliott, 2014). Coupling isotopic analysis with elemental quantification allows the detection of pollution signals even if elemental contents are low or fluctuating during the exposure period and even if a given source is a relatively minor contributor in the study area (e.g. the influence of an anthropogenic source against a prevailing natural background source).

Due to the harmful effects of the persistence of pollutants on the lichen physiology, lichen diversity tends to decline in more polluted areas (Ribeiro et al., 2010). Hence, the compositional changes in lichen communities are correlated with changes in levels of atmospheric pollution (Conti and Cecchetti, 2001). This relationship substantiates the use of lichens as ecological indicators through diversity metrics of lichen communities, based on the differential sensitivity of lichen species in response to atmospheric changes (Branquinho et al., 2015). The assessment of diversity metrics like abundance and frequency of occurrence, and specific tolerance of naturally occurring (native) lichen species, in a study area, provides an overall evaluation of the degree of atmospheric pollution, making lichens also excellent surrogates of air quality (Conti and Cecchetti, 2001; Branquinho et al., 2015). In recent years, trait-based metrics (aggregating species by functional groups sharing the same functional attributes - e.g., thallus growth form, photobiont, reproductive strategies) are increasingly applied alternatively or additionally to species richness metrics, addressing current predominant drivers of environmental change (e.g. climate change, land-use change, nutrient phosphorous or nitrogenous pollution) which often do not necessarily lead to an overall decline on the number of lichen species but rather primarily trigger shifts in species composition of lichen communities (Koch et al., 2019).

Besides bioaccumulation levels and variations in diversity, the responsiveness of lichens to environmental changes is further expressed by alterations of morphological and physiological features, revealing their functional response to environmental exposures in terms of effects upon structural, vitality and performance parameters (Augusto et al., 2007; Pinho et al., 2008). Measuring the morphological changes and/or evaluating the physiological parameters in the lichen thallus is often coupled with the quantification of bioaccumulated pollutants within the same matrix to determine the thresholds of effect or damage proportional to dose (Conti and Cecchetti, 2001; Garty, 2001). Amongst the lichen physiological parameters commonly affected by environmental pollution are photosynthesis and respiration rates, potential quantum yield of photosystem II (PSII) (F_v/F_m ratio), chlorophyl content and integrity, stress-ethylene production, cell membrane integrity, ultrastructural responses, ATP content or N fixation (Conti and Cecchetti, 2001; Garty, 2001).

Lichen biomonitoring studies in field conditions may follow three main approaches concerning the method of exposure – collection of native lichens, lichen transplantation or on-site lichen fumigation (Branquinho et al., 2008; Cecconi et al., 2019). Due to the slow rate of lichen growth and their passive residency, the sampling of native lichens is more suitable to represent the long-term, time-integrated exposure to the atmosphere characteristic of the surrounding environment (Branquinho et al., 2008; Ribeiro et al., 2010). While lichens can keep a signal of past pollution events, they also respond quickly to atmospheric changes in pollutant levels (Munzi et al., 2011; Augusto et al., 2015). Both lichen transplantation and fumigation are more suitable to study the short-term effects of air pollution, as the duration of exposure can be defined (Branquinho et al., 2008). Lichen transplantation enables biomonitoring in the absence of native lichens, with repeatability and greater spatial flexibility, enabling fine-tuned sampling designs for delimiting the area of influence around specific sources (Williamson et al., 2008; Caggiano et al., 2015). Lichen fumigation allows to control not only the duration but also intensity of exposure of the lichens and, like transplantation, to determine their pre-exposure condition (Caggiano et al., 2015).

1.4. Environmental threats at the study area (São Miguel, Azores)

São Miguel island (SMG), where this thesis was carried out, is the largest (744 km^2) of the nine volcanic islands and other islets making the Azores archipelago, located in the North Atlantic Ocean, a major submarine mountain range continuously formed by volcanic activity of the Mid-Atlantic ridge (Santos et al., 1995; Guénette and Morato, 1997). The Azores, a Portuguese autonomous region and an EU outermost region, is one of the most remote and isolated archipelagos in the Earth, at 1400 km west from the closest continental landmass of mainland Portugal (Encyclopaedia Britannica, 2021). The hallmark features inherent to small islands (<10.000 km^2, <500.000 pop.; Beller et al., 1990) define the main constraints shaping SMG distinct environmental context, namely confined territory, coastal hazards, geographic remoteness and isolation, in addition to an unstable geologic setting at the triple junction of the North American, Eurasian, and Nubian tectonic plates, posing a continuous risk of destructive volcanic and seismic events (Calado et al., 2007; Carmo et al., 2015).

Geologically, SMG presents an east-west elongated shape, comprising three quiescent central trachytic volcanoes with summit calderas (Furnas, Fogo and Sete Cidades) linked by zones of fissure volcanism (Picos and Congro), with the easternmost portion beyond Furnas being older and inactive (Viveiros et al., 2010; Carmo et al., 2015). SMG is the most volcanically active island of the Azores, with episodes of explosive eruptions in recorded history at Fogo (1563) and Furnas (1630), though the latest land eruption dates back to the XVII century (Gaspar et al., 2015). Despite the apparent serenity of a long eruptive dormancy period, the inhabitants are continually reminded of the volcanic hazard not only by the reminiscent volcanic structures that configure the island's topography and landscapes but also by regular seismic activity and several secondary volcanic manifestations (Viveiros et al., 2010; Caliro et al., 2015; Viveiros et al., 2020).

Nevertheless, human settlement has occurred in areas inside or around the calderas, such as Furnas and Ribeira Quente villages, at the dormant yet actively degassing Furnas volcano, where houses are built directly on top of hydrothermal emanations, promoting indoor gas entrapment and chronic exposure to volcanogenic emissions (Viveiros et al., 2010; Linhares et al., 2018). The populations living in these villages are certainly of the most consistently studied worldwide manifesting health effects of living under chronic exposure to volcanogenic air pollutants, namely genotoxicity and cytotoxicity of tissues of the upper respiratory tract, respiratory pathologies, and various cancers (Rodrigues and Garcia, 2015). Besides, Furnas volcano has provided a privileged natural laboratory to conduct research showcasing the effects in other organisms, from earthworms (Cunha et al., 2011) to mice (Camarinho et al., 2013, 2019). Therefore, in view of the perpetuality of non-eruptive volcanic activity, the chronicity of human exposure to the continuous emission of airborne pollutants and the potential severity of ensuing adverse health outcomes, Furnas volcano is a prominent environment motivating research efforts and raising public health concerns regarding air pollution of natural origin in the Azores (Rodrigues and Garcia, 2015). These are key reasons why Furnas volcano was targeted as a study site for experimental work in this thesis to develop biomonitoring tools more appropriate for volcanogenic emissions.

A second major environmental threat addressed by this thesis is intense land use change dynamics driving the degradation of native habitats. The disturbance caused by human colonization from the XIV century onwards resulted in a dramatic modification of SMG native habitats due to deforestation of the native forest cover (Fernandes et al., 2015), leading to the almost complete extinction of native vegetation below 500 m of altitude by 1850 (Triantis et al., 2010; Borges et al., 2019). The dense forests originally covering the island were progressively replaced for anthropogenic habitats, with present-day native vegetation cover being restricted to only 8.7% with 0.4% of native forest cover (Hortal et al., 2010; DRA, 2018; Borges et al., 2019). Preserving and managing the last remaining fragments of native habitats is currently a highly critical challenge, considering they are irrecoverable pockets of unique endemic biodiversity at risk of extinction, holding also valuable ecosystem services, recreative potential and ecotourism appeal (Borges et al., 2009; Fernandes et al., 2015).

Habitat and biodiversity loss directly related to land use changes continues to escalate given the pressure of human development, under a restricted landmass, with an extremely heterogeneous landscape and irregular topography shaping severe land use constraints (Fernandes et al., 2015; Borges et al., 2019). This is mainly driven by urban expansion and associated road networks as well as implementation of large areas of high elevation intensive pasture monocultures (Triantis et al., 2010; Borges et al., 2019). The ensuing massive application of agrochemicals for fertilization and pest control has become a major source of environmental pollution (Parelho et al., 2016; Borges et al., 2019). Reforestation with plantations of exotic tree *C. japonica* and the introduction and uncontrolled spreading of exotic and invasive species are aggravating factors jeopardizing the preservation of the already overwhelmed natural habitats (Fernandes et al., 2015). Tree plantations provide little room for diverse understory vegetation and are overtaking native forests at higher elevations, while invasive species dominate over the vulnerable native island biota, leading to biodiversity loss by resource and territorial competition (Borges et al., 2009, 2019). Notoriously, invasive species of vascular plants such as *Pittosporum undulatum*, *Hedychium gardnerianum*, *Gunnera tinctoria*, *Clethra arborea*, *Acacia melanoxylon* or *Eucalyptus globulus* have aggressively colonized the whole island (Silva et al., 2008; Borges et al., 2009; Hortal et al., 2010; Hanski, 2016).

Another threat for the environmental context of SMG, like the generality of small oceanic islands, is the high vulnerability to the impacts of global climate change mediated by anthropogenic air pollution, which is already posing problems on multiple dimensions, including hydrological cycles, weather patterns, vegetation cover and forest structure, biodiversity erosion, availability of energy and natural resources, yields of farming and fisheries and coastal erosion (Calado et al., 2011; DRA, 2015, 2017; Sieber et al., 2018; DRA, 2019). Insular territories are acutely susceptible to effects of climate change upon hydrological processes regulating freshwater availability. Freshwater reserves in oceanic volcanic islands are limited and have a short residence time, undergoing continuous natural discharge and suffering quality decay at an accelerated rate, particularly due to saltwater intrusion, which is facilitated by coastal erosion (DRA, 2015). Thus, the influence of climatic conditions upon water supply from atmospheric precipitation and water loss through surface evapotranspiration is critical for hydric reserves at surface water bodies as well as subsoil infiltration and retention at underground water reservoirs (Borges et al., 2009; DRA, 2015). Current meteorological data and climatic projections for the Azores predict a progressive decline of precipitation coupled with greater maximum air temperatures leading to drier summers (DRA, 2015; Borges et al., 2019). Freshwater scarcity is bound to become a major constraint to which the region must adapt, as evidenced in the summer of 2018, when an unusual severe drought caused considerable agricultural losses (DN, 2018).

On the other hand, the frequency of episodes of extreme rainfall is also likely to increase due to the elevation of the surface temperature of the ocean, potentially triggering landslides and flooding episodes (DRA, 2015). End cycle hurricanes and tropical storms, which in the past used to dissipate at higher latitudes, have been surviving longer (DRA, 2015), like the most recent hurricane Lorenzo, costing 330 million € in damages across multiple Azorean islands (AO, 2020). Although projections for sea level rise are milder due to the high coastlines and steep slopes typical of volcanic islands, the combination with extreme weather phenomena and seismic activity aggravates coastal erosion. Small islands are especially vulnerable to this process because the narrow coastal fringe holds most of settlement and economical potential and relocation of infrastructures is conditioned by limited land availability (Calado et al., 2011; DRA, 2015).

Island biodiversity is inherently vulnerable to climatic shifts disrupting seasonal weather patterns and the occurrence of extreme weather events. The ensuing alteration of native habitats can ultimately result in a cascade of local extinctions if species are displaced from their suitable elevational and latitudinal ranges and are left with no colonization options (Sieber et al., 2018; Borges et al., 2019; Veron et al., 2019). Species dependent on the hyper humid conditions of the last remaining fragments of high elevation native forests of the Azores are further endangered by the prospects of drought (Borges et al., 2019). Overall, the conjugation of these natural and anthropogenic threats, acting at both global and local scales, are creating substantial challenges which, unless dealt with appropriately in a perspective of sustainable development, will lead to the irreversible degradation of the local environment and consequently worsen the quality and cost of life of the local population.

Thesis hypotheses, main objective, and outline

The **rationale** behind this thesis is addressing the threat posed by air pollution from intensifying anthropogenic practices and volcanic activity to ecosystems and human health in the context of volcanic islands such as the Azores, proposing and demonstrating the use of established biomonitors of air pollution, such as lichens, as suitable tools to estimate the deposition of airborne emissions in the studied areas. The final outputs are meant to provide scientific bases evidencing the usefulness of integrating biomonitoring tools and methods in: i) environmental monitoring and environmental impact assessments; ii) assessing human health risks from exposure to volcanic and anthropogenic air pollution; iii) guiding policies of sustainable development and of public health protection against natural hazards in volcanic islands. The work conducted in this thesis has a case study applied to the Azores, further highlighting them as a privileged location to conduct research on topics such as the impacts of active volcanism, drivers of environmental change, ecological indicators, and ecological preservation. This choice is motivated by the expediency of such topics from a regional standpoint, particularly considering the current legislative, societal, and scientific goal of harmonizing socioeconomic progress with the preservation of excellent air quality under the ecological vulnerability inherent to smaller oceanic islands.

Bearing in mind these intentions, the four **main hypotheses** of this thesis are:

- Lichens are able to trace the long-term pattern of airborne dispersal of hydrothermal emissions in a degassing volcanic area;
- This ability allows to spatially model the risk areas of chronic exposure to hydrothermal air pollution with high resolution and time-integration;
- Green-algal lichens are bioindicators of elevated CO_2 in natural environments by responding with significant thalli structural changes following the prolonged exposure to hydrothermal degassing;
- The elemental bioaccumulation profile of native lichens displays that agricultural and artificial land uses are significantly more impacted by airborne pollution than forest areas in São Miguel island.

These hypotheses lead to the **main objective of the thesis**: develop and apply the use of lichens as biomonitors of volcanogenic and anthropogenic air pollution occurring in volcanic islands, supporting the implementation of air pollution biomonitoring programs therein.

The **outline** of the thesis (Figure 1) is structured according to the following sequence of main chapters. **Chapter 1** consists of a general introduction providing a supportive background to elucidate the pertinence of this thesis, primarily framed in the context of the current environmental challenges faced by volcanic oceanic islands like the Azores. The hypotheses, objective and outline of the thesis are described at the end of this first chapter. **Chapter 2** substantiates, through the use of native lichens as long-term biomonitors, the significant polluting impact resultant from the currently intensifying agricultural and urban/industrial activities carried out in São Miguel island. This work reveals that the anthropogenic disturbance on human impacted areas is reaching such an extent that noticeably deviates from the pristine-like state of the island, whose preservation requires ongoing controlling and monitoring measures. **Chapter 3** deals with the co-existing menace of air pollution from natural origin in São Miguel, using transplanted lichens as biomonitors of hydrothermal volcanic emissions at the actively degassing and inhabited Furnas volcano. It is divided in two sub-chapters, each corresponding to an individual publication. **Sub-chapter 3.1.** demonstrates the time-integrated ability of lichens to trace airborne hydrothermal emissions and subsequently applying it to develop a novel methodology of spatially modelling the risk areas of chronic exposure for the population. **Sub-chapter 3.2.** demonstrates the sensitivity of lichens as ecological indicators of the continuously degassing environment of Furnas volcano, validating a set of thallus structural alterations as biomarkers reflecting the prolonged exposure to elevated CO_2 levels. Finally, **Chapter 4** contains an overall conclusion wrapping up the thesis, with a few remarks on future perspectives regarding the importance of implementing air pollution biomonitoring tools in regular environmental surveillance in the Azores, as well as some critical discussion on the limitations and future opportunities of research stemming from the studies of the thesis.

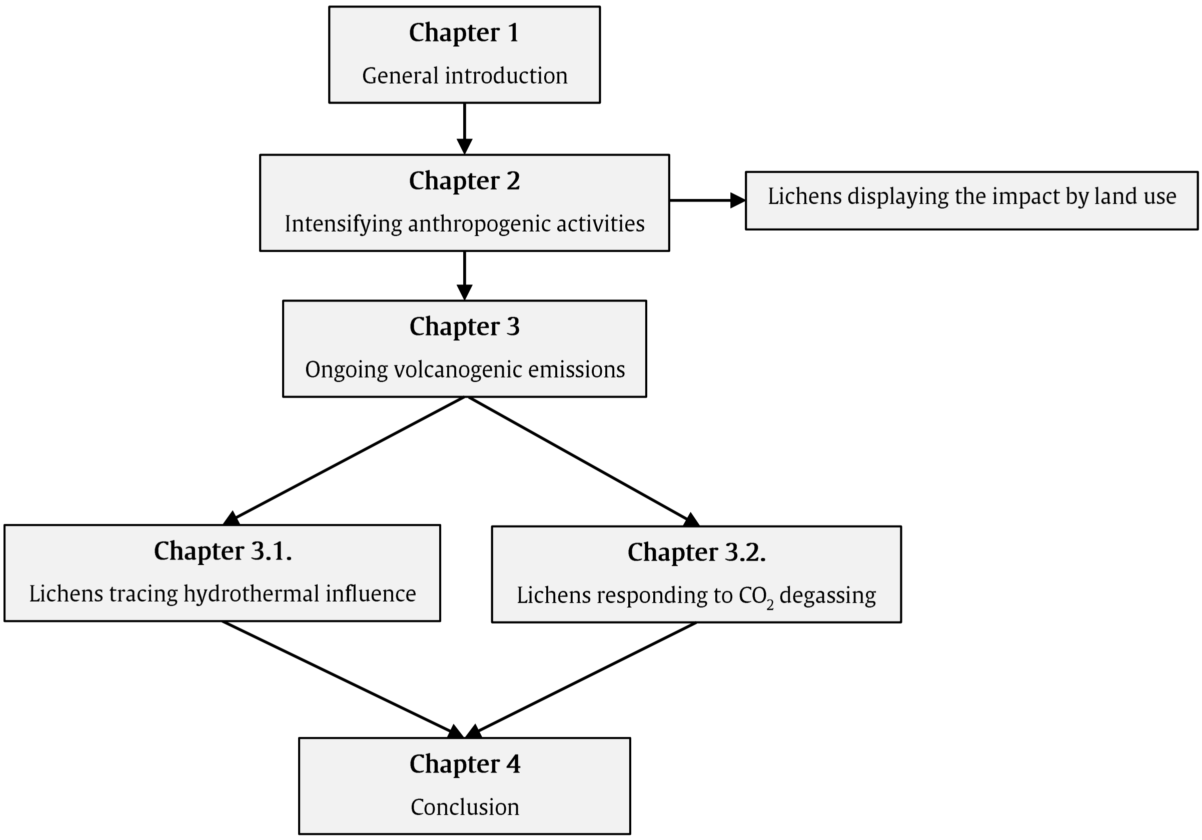

Figure 2: Diagram illustrating the outline of the thesis and the sequence of the chapters.

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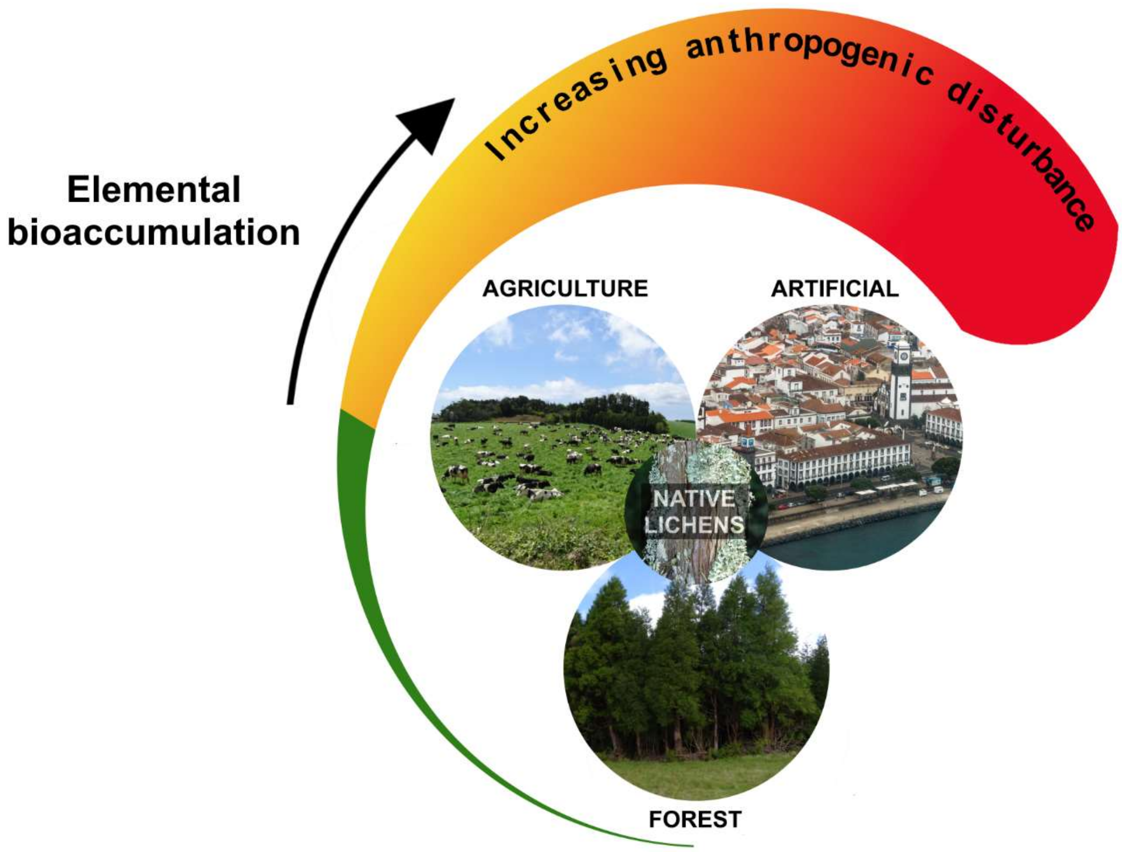
CHAPTER 2

Lichens as biomonitors of anthropogenic impact by land use



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Elemental profile of native lichens displaying the impact by agricultural and artificial land uses in the Atlantic island of São Miguel (Azores)

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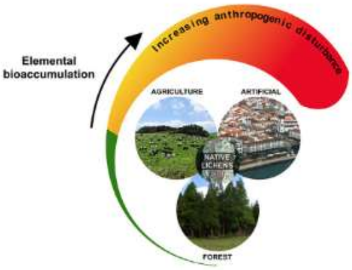
HIGHLIGHTS

- Native lichens displayed elemental enrichment along an island's land use gradient.
- Bioaccumulation ratios reached high levels in artificial sites.
- %N was higher with distinctly lower $\delta^{15}N$ isotopic signal in agricultural sites.
- This significant anthropogenic impact threatens the pristine environment.

GRAPHICAL ABSTRACT



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ABSTRACT

Smaller oceanic islands, often hosting endangered native habitats, are particularly vulnerable to the impact of human activities. Using lichens as bioindicators, this study aimed to test if agricultural (AGR) and artificial (ART) land uses are noticeably more impacted than forest (FOR) land use on an oceanic island (São Miguel, Azores). Livestock and farming practices in AGR areas involve the intensive application of synthetical agrochemicals as well as organic fertilizers and manure. ART areas accommodate vehicular traffic besides industries dedicated to waste management, energy production or exploration and transformation of raw materials. Naturally occurring *Parmotrema* lichens were collected in 28 sampling sites distributed between each land use. The concentrations of 58 elements as well as the percentage (%N) and the isotopic composition of nitrogen ($\delta^{15}N$) were determined on lichen samples. An overall pattern of significant elemental enrichment was observed in lichens from AGR and ART sites compared with FOR lichens, including several rare-earth elements. FOR lichens were noticeably cleaner, thus providing background concentrations for the calculation of bioaccumulation ratios. Bioaccumulation levels were generally low to moderate in AGR lichens and moderate to high in ART lichens, including toxic heavy metals. %N was highest in AGR lichens and its isotopic signature was distinguishable from ART lichens by significantly lower $\delta^{15}N$ values. This study provides a comprehensive baseline of bioaccumulation data across major land uses for comparison with other insular regions, highlighting the

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Abstract

Smaller oceanic islands, often hosting endangered native habitats, are particularly vulnerable to the impact of human activities. Using lichens as bioindicators, this study aimed to test if agricultural (AGR) and artificial (ART) land uses are noticeably more impacted than forest (FOR) land use on an oceanic island (São Miguel, Azores). Livestock and farming practices in AGR areas involve the intensive application of synthetical agrochemicals as well as organic fertilizers and manure. ART areas accommodate vehicular traffic besides industries dedicated to waste management, energy production or exploration and transformation of raw materials. Naturally occurring *Parmotrema* lichens were collected in 28 sampling sites distributed between each land use. The concentrations of 58 elements as well as the percentage (%N) and the isotopic composition of nitrogen ($\delta^{15}N$) were determined on lichen samples. An overall pattern of significant elemental enrichment was observed in lichens from AGR and ART sites compared with FOR lichens, including several rare-earth elements. FOR lichens were noticeably cleaner, thus providing background concentrations for the calculation of bioaccumulation ratios. Bioaccumulation levels were generally low to moderate in AGR lichens and moderate to high in ART lichens, including toxic heavy metals. %N was highest in AGR lichens and its isotopic signature was distinguishable from ART lichens by significantly lower $\delta^{15}N$ values. This study provides a comprehensive baseline of bioaccumulation data across major land uses for comparison with other insular regions, highlighting the greater vulnerability of island ecosystems to anthropogenic impacts even if by relatively small-scale human activities.

Highlights:

- Native lichens displayed elemental enrichment along an island's land use gradient.
- Bioaccumulation ratios reached high levels in artificial sites.
- %N was higher with distinctly lower $\delta^{15}N$ isotopic signal in agricultural sites.
- This significant anthropogenic impact threatens the pristine environment.

Keywords: Island ecosystems; Land use; Native lichens; Air pollution; Trace elements; Bioaccumulation ratio.

1. Introduction

Due to their remoteness, isolation, confinement, complex orography, restricted land availability and limited supply of natural resources, smaller oceanic islands are particularly vulnerable to natural and anthropogenic drivers of environmental change (Borges et al., 2009; Scandurra et al., 2018; Calado et al., 2018). Harmonizing anthropogenic activities with the ecological vulnerability of island native habitats is highly challenging as human pressure easily surpasses sustainable limits (Borges et al., 2009). Throughout history, episodes of colonization and settlement at newfound islands, such as the Azores archipelago, have showcased this challenge, resulting in dramatic and irreversible transformation of native ecosystems, host to unique and increasingly endangered endemic biodiversity (Borges et al., 2019). Thus, small island ecosystems are even less resilient to rapid and extensive land use changes, one of the most impacting anthropogenic drivers (Borges et al., 2019; Xie et al., 2019; Massetti and Gil, 2020). A primary avenue of land use change corresponds to deforestation of pristine forest habitats to exploit natural resources and make way for farming and livestock (Borges et al., 2009, 2019). Volcanic islands in particular have naturally fertile soils which are attractive for agricultural purposes (Parelho et al., 2014). Another major avenue of land use change with special relevance for traditionally agro-rural insular regions is urbanization driven by growing touristic interest, with the spreading of residential areas and related infrastructures, such as roads, industries, or waste management facilities (Calado et al., 2018; Borges et al., 2019). Monitoring the environmental impact caused by these anthropogenic activities linked to land use change becomes imperative considering the vulnerability of island ecosystems.

Lichens are exceptional ecological indicators that are widely used to evaluate the effects of human activities on ecosystem structure and functioning, for instance through elemental bioaccumulation metrics (Branquinho, 2001; Branquinho et al., 2015). Some peculiar traits confer to lichens their distinctive suitability as bioindicators that reliably reflect the level of airborne environmental contamination (McGeoch, 1998). Lichens, as non-parasitic poikilohydric epiphytes, depend entirely on the atmosphere rather than the substrate to obtain water and mineral nutrients (Branquinho et al., 2015). Devoid of roots, cuticle or stomata to regulate nutrient and water exchange, the entire surface of the symbiotic thallus continually absorbs airborne elements in a mostly passive and indiscriminate fashion, even if present in vestigial amounts, allowing the quantification of several elements within the same biological matrix. Coupled with their sessility and slow growth, naturally occurring (native) lichens can retain a time integrated record of the elemental and isotopic composition of the surrounding atmosphere (Conti and Cecchetti, 2001; Garty, 2001; Wolterbeek, 2002; Batts et al., 2004; Williamson et al., 2008; Backor and Loppi, 2009; Bernardo et al., 2019). Adequately interpreted lichen bioaccumulation data is invaluable in environmental forensics by uncovering disrupting factors, thus aiding governing authorities in the implementation of corresponding corrective and mitigating measures towards environmental protection (McGeoch, 1998; Branquinho et al., 2015; Cecconi et al., 2019).

Anticipating that native lichens maintain equilibrium with the surrounding environment, expressing the specific composition of airborne pollutants resulting from emissions of diverse natural processes and anthropic activities linked to different land uses (e.g. Pinho et al., 2008; Koch et al., 2016; Port et al., 2018), this study aimed to test if agricultural and artificial land uses are noticeably more impacted than forest areas in the context of a smaller oceanic island. For that purpose, the island of São Miguel (Azores, Portugal) was selected for a broad elemental survey on native lichens naturally occurring on agricultural, artificial and forest areas, the latter providing a pristine background in a geographic setting remotely located from major centers of air pollution.

2. Materials and methods

2.1. Study area

With 744.55 km^2 of area and a population of just under 140.000 inhabitants, São Miguel island (SMG) is the largest and most populated of the Azores archipelago, in the north Atlantic Ocean. The island stretches at approximately 66 km of length by 16 km of width, with the highest peak at 1108 m above sea level and is notoriously characterized by a variety of volcanic structures, including three central active trachytic composite volcanoes with caldera (Sete Cidades, Fogo and Furnas) (DRA, 2015). According to the latest regional land use survey, published in 2018 (DRA, 2018) (Fig. 1), mostly based on orbital data by *Satellite Pour l'Observation de la Terre* [SPOT], over half of SMG territory (58.95%) is occupied by agricultural land, majorly comprised of permanent meadows and pastures with herbaceous vegetation coverage for cattle grazing (47.07%). Another third of SMG territory (31.08%) is occupied by forest terrain, dominated by angiosperm (13.66%) and gymnosperm (9.48%) forests. Artificial land use amounts to 6.02%, including urban areas (3.89%), mostly coastal, as well as industries and traffic (1.29%), mining and waste management (0.47%) and urban green spaces and recreational parks (0.37%). This tripartite land use follows an altitudinal gradient with urban areas established near the coast, forest areas filling the elevated landscapes and agricultural areas in between, spreading throughout the entire island except at the peak altitudes. SMG enjoys a temperate oceanic climate, generally characterized by mild temperatures, low thermal amplitude, high pluviosity, constantly high air humidity and persistent winds, predominantly from west quadrant (DRA, 2017).

In recent years, agricultural and touristic activities have been intensifying in the region, which is reflected in the increase of the resulting emissions of atmospheric pollutants. Since 1990, overall data available for the Azores archipelago shows that fossil fuel combustion, mainly for transportation and electricity, has contributed with a 73% increase of CO_2 and 45% of CH_4 emissions (DRA, 2019a). Concurrently, agriculture, encompassing mostly bovine cattle raising and farming, has contributed with an 82.7% increase of CO_2 and 93% increase of CH_4 emissions (DRA, 2019a). Land use for agricultural purposes involves the intensive application of synthetic agrochemicals, including inorganic fertilizers and pesticides, as well as organic fertilizers and manure, resulting in pollution by phosphates, ammonia, nitrogen oxides, and heavy metals. Meanwhile, artificial land use accommodates fuel combustion by vehicular traffic and thermoelectric power generation besides various industries in waste management and incineration, food processing, inert extraction, and transformation of raw materials in metalwork, woodwork, concrete, and cement. Collectively, these are noteworthy for releasing ammonia, nitrogen and sulfur oxides and particulate matter along with heavy metals (Parelho et al., 2014; DRA, 2019a).

2.2. Sampling design

The tripartite land use gradient was the basis for the sampling design. The sampling sites chosen for the retrieval of *in-situ* naturally occurring epiphytic lichens were representative of each major hierarchy of land use defined in the latest land use survey of the Azores (DRA, 2018). In total, twenty-eight suitable locations were chosen in SMG island, ten agricultural (AGR), ten artificial (ART) and eight forest (FOR) (Fig. 1).

The strategy for site selection was very conditioned by two main criteria: altitude and land use predominance. Due to the altitudinal gradient described in section 2.1, altitude was restricted at 700 m to reduce the influence of weather variation with elevation, especially of precipitation, avoiding the highest levels of precipitation reaching above 3000 mm at 1000 m of elevation. Near sea level, mean total annual precipitation is around 1000 mm, mean annual relative humidity ranges between 74% and 84% and mean annual temperatures range between 14.2 ºC and 20.4 ºC (DRA, 2015), which do not pose an extreme contrast of arid and dry conditions and should rarely become limiting for lichen physiology, with frequent days of rainfall occurring throughout most of the year. At mid and low elevation, the island is extensively occupied by a heterogenous mosaic of pastures and cultivation fields with delimiting vegetation (Borges et al., 2019). Consequently, land use continuity of ART areas and FOR areas at lower altitude is limited to short distances before shifting back to AGR land use. After measuring the percentage of area occupied by each land use on differently sized circular buffers surrounding potential sampling areas, it was determined that a 200 m radius buffer is the lengthiest that could be set to define sampling sites with consistently at least 50% of area occupied by either ART or FOR land use at lower altitudes. Additionally, it was attempted to spread sampling sites evenly over the island to incorporate spatial variability and to stick with a single phorophyte tree species, *Cryptomeria japonica* being the obvious candidate as the most widespread. However, considering the relatively small area of the island area occupied by ART land use (6.02%), some exceptions were made: a) ART sampling sites were spatially confined to the most urbanized zones where there is greatest ART land use continuity and spatial convergence of traffic and industrial activities, with a minimum distance of 500 m between buffer borders; b) since *C. japonica* is scarce in ART areas (and no other tree species is as widespread), other available trees found within otherwise suitable ART land use buffers were considered; c) since industrial facilities are often interspersed by agricultural terrains, if no other choice was available, the predominance of ART land use was exceptionally set at 50% on a smaller 100 m buffer (this was the case for two of ten chosen ART sampling sites).

Other factors further diminished the pool of sampling sites and limited their spatial distribution after on-site verification, such as: a) inaccessibility by natural barriers and private property; b) suspicion of ongoing human activities nearby forest areas; c) absence of trees or absence of lichen epiphytic colonization on tree trunks due to natural factors (shielding from sunlight due to unpruned dense canopy of *C. japonica* or obstruction by shrubby vegetation, obstacles, and other trees). In denser forest areas, lichen epiphytic colonization was limited to the less shaded outermost trees, excluding from sampling most of the forest area represented in the land use survey.

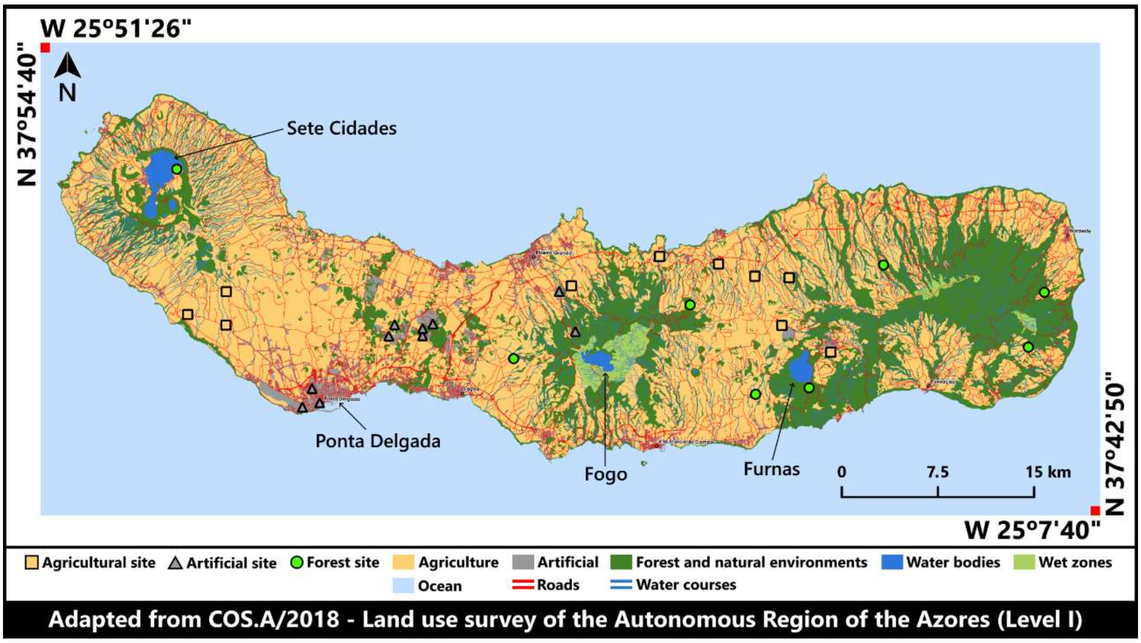

Figure 1. Location of the 28 sampling sites in São Miguel island according to the latest 2018 land use survey (Level I) of the Azores (COS.A / 2018) (DRA, 2018). Sete Cidades, Fogo and Furnas calderas are shown, as well as the main city of Ponta Delgada.

2.3. Lichen collection

The collection of native lichens from sampling sites occurred during the second half of 2019. As none of the sampling sites underwent land use change for at least a decade or more, the collection of thalli of *in-situ* native lichens guaranteed an exposure for a long period to the air composition in each site, thus providing temporal integration of the elemental deposition characterizing each land use. A composite sample was obtained from each site, comprising lichen thalli plucked from a group of 3 to 5 adjacent trees to reduce variability within site. Only healthy, living trees with straight and unobstructed trunks at 1 m to 3 m of height were considered.

Foliose lichens of the genus *Parmotrema* were selected for collection primarily because it is of the few macrolichen genera occurring ubiquitously in all sites across the three land uses, likely owing to the tolerance of many *Parmotrema* species to environmental disturbances, such as nitrogen eutrophication and sulfur acid deposition (USFS, 2020). Also, large foliose lichens with thin flat thalli, such as *Parmotrema* species, not only allow simple collection, easy handling and processing, but also have greater capability to accumulate metal-containing airborne particles due to their high ratio of exposed surface area to dry weight (Garty, 2001). However, it was not possible to find the same *Parmotrema* species occurring across all sampling sites. Overall, three species were collected: *P. reticulatum* exclusively in all ART sites and either *P. reticulatum*, *P. perlatum* or *P. robustum* in AGR and FOR sites. The identification of species followed the *Parmotrema* dichotomic key by Schumm (2008) for lichens of Madeira, Canary Islands and Azores. These three species are the most alike while also having wide distribution and occurrence in each of the three major land uses in SMG island (Azorean Biodiversity Portal, 2020). Interspecific variations in elemental bioaccumulation are minimized considering their morphologically similar thin flat thalli and corresponding ratio of surface area to dry weight, the most relevant morphological determinant of lichen elemental content (Garty, 2001). On a previous study involving 8 sampling sites of two other Azores islands (Santa Maria and Terceira), *P. bangii*, *P. crinitum* and *P. robustum* showed rather low interspecies variability as to biological levels of microelements, confirming that the response of various species within the same genus, sharing common morphological features, is irrespective of the species itself (Vieira et al., 2007). Furthermore, *P. reticulatum* and *P. perlatum* are both classified as eutrophic (tolerate and are often enhanced by N deposition loads above 8.0 kg N/ha/yr) and tolerant to acid deposition (unaffected by S deposition at or above 8.0 kg S/ha/yr) (USFS, 2020) and it is likely that *P. robustum* is as tolerant given its occurrence in urban and intensive agriculture areas, further displaying the similarity of these species.

More frequently than not, only one species was occurring in each sampling site. Where two or more *Parmotrema* species were coexisting on the same site, one species would often predominate over others in terms of epiphytic colonization. Therefore, the most abundant species occurring in each site was collected. In total, on AGR land use, *P. reticulatum* was collected in 2 sites, *P. perlatum* in 5 sites and *P. robustum* in 3 sites. On FOR land use, *P. reticulatum* was collected in 1 site, *P. perlatum* in 3 sites and *P. robustum* in 4 sites. *P. reticulatum* was collected in all 10 ART sites. Lichen thalli were retrieved at a height of at least 1 m above ground to ensure predominantly atmospheric rather than soil influence. A composite sample of 5 g was obtained per site, containing a balanced mixture of thalli from the 3 to 5 selected trees. A uniform sampling approach by weight rather than thalli size or thalli number was applied because *Parmotrema* lobes would often cover extensively the tree trunks without clear delimitation of individual thalli from margin to margin. As land use is unchanged for a decade or more in all sampling sites, large sections of thalli were taken, several cm deep from the periphery towards the center, thus accounting for a significant fraction of the lichens' lifetime. Only healthy-looking thalli were collected, as clear as possible from other lichens, algae, and mosses or other materials. Damaged or obstructed portions were excluded. Once retrieved from the field, all lichen samples were oven dried at 40 ºC for 72 h to better preserve them for storage. No preliminary water cleansing was made to avoid washing away a substantial elemental load that would heavily distort the genuine elemental concentrations bioaccumulated in field conditions. After clearance of extraneous material, such as tree bark, the lichen samples were sealed and stored until analyzed.

2.4. Laboratory analytical procedures

The isotopic composition of N ($\delta^{15}N$) was determined on dried macerated lichen samples by continuous flow isotope-ratio mass spectrophotometry (CF-IRMS) (Preston and Owens, 1983) for vegetation matrices (LIE-SIIAF, 2020). More specifically, analyses of $\delta^{15}N$ were carried out on a Sercon Hydra 20-22 (Sercon, UK) stable isotope ratio mass spectrometer, coupled to a EuroEA (EuroVector Italy) elemental analyzer for online sample preparation by Dumas-combustion. Nitrogen delta (δ) was calculated by $\delta = [(R_{sample} - R_{standard})/R_{standard}]$, expressed in ‰ (per mil) units, where R is the ratio of $^{15}N/^{14}N$ stable isotopes. Analytical quality control for $\delta^{15}N$ was ensured by the analysis of two primary reference materials: USGS-35 with $\delta^{15}N_{Air}$ = 2.70 ± 0.20‰; casein protein with $\delta^{15}N_{Air}$ = 5.84 ± 0.1‰. A secondary reference material, Wheat Flour Standard (WFS) OAS (with $\delta^{15}N_{Air}$ = 2.79 ± 0.03‰) (Elemental Microanalysis, UK), was checked against these primary reference materials (Coleman and Meier-Augenstein, 2014). $\delta^{15}N_{Air}$ refers to air. Measurement accuracy was checked by the analysis of 9 replicates of the secondary isotopic reference material WFS OAS, interspersed amongst the sample batch, from which analytical variance was calculated with standard deviation of 0.09‰ for $\delta^{15}N$. The major mass signals of N were used to calculate total N abundance using WFS OAS as elemental composition reference material with 1.47% N (LIE-SIIAF, 2020).

The determination of the concentrations of the following 58 elements was made by inductively coupled plasma mass spectrometry (ICP-MS) for vegetation matrices on a laboratory accredited to standards ISO/IEC 17025:2017 and ISO 9001:2015 (Actlabs, 2020): Ag, Al, As, Au, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Dy, Er, Eu, Fe, Ga, Gd, Hf, Hg, Ho, In, K, La, Li, Lu, Mg, Mn, Mo, Na, Nb, Nd, Ni, P, Pb, Pd, Pr, Pt, Rb, Sb, Se, Sm, Sn, Sr, Ta, Tb, Th, Ti, Tm, U, V, W, Y, Yb, Zn and Zr. After maceration in a Retsch cutting mill with internal 1 mm sieve, raw dried samples underwent aqua regia "partial" digestion at 95 ºC for 2 h, a method using a combination of concentrated hydrochloric and nitric acids to dissolve the samples and induce elemental leaching for subsequent analysis and quantification. Resultant sample solutions were diluted and analyzed on a PerkinElmer Sciex ELAN 6000, 6100 or 9000 ICP/MS. Analytical quality control was ensured by the analysis of two certified reference materials for elemental composition, NIST 1575a and CDV-1, as well as one blank and two duplicates (two independent measurements on two different samples) per element for the batch of 28 samples. The instrument was also recalibrated once. Measurement accuracy was checked by performing the analysis of the certified reference materials along with the sample batch, from which the percentages of recovery were determined against the certified elemental concentrations specified in the certificates of analysis of the reference materials. 18 elements had no certified concentrations for the reference materials and hence their recoveries could not be calculated (Table S4). The percentages of recovery and the individual values of blanks and duplicates per element are available as Table S4 of Supplementary material. For statistical calculations, concentration values below the lower detection limit were considered as equal to that limit.

2.5. Statistical analysis

Priorly to any statistical computations, extreme outlier values of all variables were detected and removed with Tukey test under a IQR (inter-quartile range) rule with a multiplier of 3. Subsequently, this required the complete removal of a sample retrieved from the vicinity of a quarry from further analysis due to extremely increased values for almost all variables (see table S3 of supplementary material), as the intent was to compare land uses rather than individual sites. The differences in mean values of the elemental concentrations and $\delta^{15}N$ isotopic values between land use groups were checked by one-way ANOVA followed by Tukey's HSD post-hoc test to determine the specific differences between groups. Sampling sites are statistical replicates of each land use group as only one single composite sample was obtained per site. Whenever the assumption of normality could not be met by Shapiro-Wilk test, the non-parametric Kruskal-Wallis H test was employed instead. Mean values of normally distributed data are reported throughout the text followed by standard error of mean (mean ± SEM), while median values of non-normally distributed data are reported followed by median absolute deviation (median ± MAD). The levels of statistical significance were set at $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***). Detailed descriptive statistics are provided in table S2 of supplementary material. Additionally, Figure S2 of supplementary material contains the differences in mean values of the percentage of carbon %C and $\delta^{13}C$ isotopic values in lichens between land uses. Lichen bioaccumulation ratios (*B* ratios) for AGR and ART land uses were determined in accordance with the method recently proposed by Cecconi et al. (2019) for native lichen samples. The elemental concentrations from the AGR and ART land uses were divided by the corresponding FOR concentrations, considered as the background element concentrations (BECs) in the context of this study, since by definition, BECs are those measured in lichen samples reflecting proximate-natural, unaltered conditions. The five-class interpretative scale proposed by Cecconi et al. (2019) was used as the authors state it is based on a robust conceptual framework (with a high sample size of n = 3773, which is not available for the Azores), and may be easily implemented in other countries. The mean values of *B* ratios plotted in Fig. 4 and corresponding 95% confidence intervals are provided in table S5 of supplementary material. A principal component analysis (PCA) was carried out to explore the pattern of correlations between variables and with land uses. The bidimensional component plot in rotated space and variable loadings for each principal component are provided in supplementary material as Figure S1 and Table S1, respectively. Statistical analysis was done with IBM SPSS Statistics v.25 (IBM Corp, 2017) and latest Microsoft Office Excel (Microsoft Corp, 2020).

3. Results and discussion

3.1. %N and $\delta^{15}N$

Significantly higher average concentrations of %N were found in lichens from AGR (2.222 ± 0.165, $p \leq 0.001$) and ART (2.081 ± 0.112, $p \leq 0.001$) sites in comparison with FOR lichens (1.051 ± 0.113) (Fig. 2). FOR values ranged between 0.647% and 1.413%, with only one non-forest site presenting a value (1.074%) lower than the forest maximum. The group of AGR lichens reached the highest %N values (2.947%) and crossed the threshold of moderate bioaccumulation ratio (Fig. 4). Slightly lower %N values were quantified on ART lichens, which remained just below the moderate threshold (Fig. 4), having almost twice as much %N on average than FOR lichens. Meanwhile, the isotopic composition of N, provided by $\delta^{15}N$, significantly differs between AGR (-11.158 ± 0.723) and ART (-6.228 ± 1.245) land uses ($p \leq 0.01$), with FOR lichens retaining an intermediate $\delta^{15}N$ signature within the range of the other two land uses (-9.235 ± 0.512) (Fig. 2). Overall, $\delta^{15}N$ values are more negative on AGR lichens, with only one site reaching above -9.2‰, and become less negative on ART lichens, leading up to the only positive $\delta^{15}N$ value observed (+0.054‰).

These results not only reflect the noticeable contribution of anthropogenic activities in AGR and ART land uses as sources of N emissions but also reveal that the $\delta^{15}N$ signature in native lichens was a suitable tracer to distinguish between each. In this regard, a consistent agreement was found with the study by Pinho et al. (2017), similarly comparing land-cover types by N concentration and isotopic composition measured in *Parmotrema hypoleucinum* on a study area with industrial facilities and intensive agriculture. These authors also reported higher %N associated with intensive agriculture and urban areas, as well as a more negative $\delta^{15}N$ signature on intensive agriculture than urban land cover, thus likewise discriminating the specific contribution of the two prevailing terrestrial N sources. In the current study, even higher %N concentrations were found in lichens from AGR (+0.83%) and ART (+0.79%) land uses. This is particularly worrisome since intensive permanent or semi-permanent pastures for cattle raising and heavy use of fertilizers have led to eutrophication of lakes and ponds and nitrate contamination deteriorating freshwater quality in SMG (Dentinho et al., 2008; Cruz et al., 2015).

Moreover, $\delta^{15}N$ results also suggest that, in this study, lichens reflected mainly ammoniacal N deposition, displaying their greater affinity to assimilate positively charged reduced nitrogen forms (NH_x) rather than negatively charged oxidized forms (NO_x) (Pinho et al., 2017). The $\delta^{15}N$ mean value obtained for AGR land use closely matched the range of values previously reported in lichens collected from areas with high cattle density (-10‰ to -8‰) (Boltersdorf and Werner, 2014), where ample loads of N in the form of ammonia are emitted from animal waste. In turn, and according to Felix et al. (2013), ammonia emitted by industries or traffic tends to relatively higher $\delta^{15}N$ values than agricultural activities. This was also verified in our study, with $\delta^{15}N$ mean value for lichens from ART land use being significantly higher than AGR. Particularly, $\delta^{15}N$ values from sites located near busy traffic roads corresponded to the characteristic $\delta^{15}N$ range of ammonia emitted by vehicles (-5‰ to -3‰) rather than traffic NO_x (+5‰ to +10‰) (Felix and Elliott, 2014). Finally, it is plausible that the $\delta^{15}N$ signature observed in FOR lichens was influenced to some extent by diffuse N deposition, leaning towards AGR values. Such influence can be explained by the selection of FOR sites that avoided the higher altitudes and was limited to intermediate altitudes (see section 2.2 - Sampling design), which are extensively occupied by pasturelands.

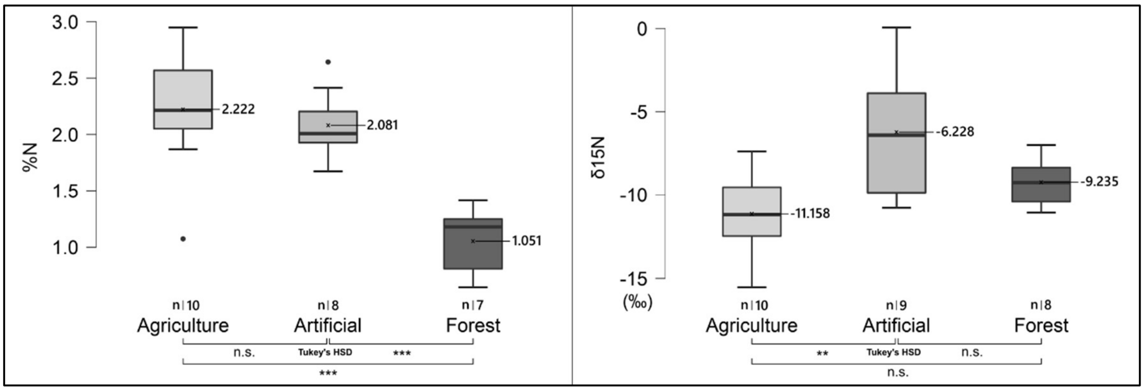

Figure 2. Boxplots displaying nitrogen concentration (N, %) and isotopic composition ($\delta^{15}N$, ‰) values measured in native lichens from the three major land uses of SMG. Mean values of each group are provided at the right side of each box. Pairwise comparisons of means are shown, and significant differences flagged at $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***) (Tukey's HSD).

3.2. Elemental bioaccumulation

An overall pattern of elemental enrichment was observed in lichens from AGR and ART sites compared with FOR lichens. Except for Rb and Hg, all other 57 elements analyzed (including N) obtained greater mean concentrations in both AGR and ART land use groups than FOR group. Besides, Hg, P and Se were the only elements which did not have at least one significantly higher mean value for either AGR or ART or both groups (at $p \leq 0.05$ level) above the corresponding FOR mean value (Fig. 3). Lichens from ART land use were more impacted by elemental bioaccumulation than AGR lichens. Only 9 of 59 elements (Hf, Hg, N, Nb, Pt, Se, Ta, U, Zr) had greater mean values in AGR than ART and of these, only Hg was significantly higher in AGR lichens. Furthermore, 54 of 59 mean elemental concentrations, except Hg, P, Pt, Se, Ta, were significantly increased in ART land use compared with FOR, while only 29 of 59 elements were significantly increased in AGR over FOR land use (Fig. 3) (Table S2). Consequently, native lichen bioaccumulation ratios were generally higher in ART than AGR land use (Fig. 4). No elements reached a severe level of bioaccumulation in AGR lichens (>4.9), only Zr reached a high level (>3.4) and 30 other elements were moderately bioaccumulated (>2.1), while in ART lichens, Sb, Sn, Bi, Co, Ni, Ba, Cu, Cr, Ti, Ag, Fe (11 of 59 elements) were severely bioaccumulated and Pb, Mo, Mn, Ca, V, Eu, Pd, Sr, Zn, La, Pr, Ce, Nd, Sm, Zr, Mg, W, Tb, Gd, Be, Y, Ho, Dy, Th, Hf (25 of 59 elements) were highly bioaccumulated, with 11 other elements moderately bioaccumulated. Elemental depletion (<1.0) occurred only for Hg in ART lichens (0.785) and Rb in AGR lichens (0.995) (Fig. 4).

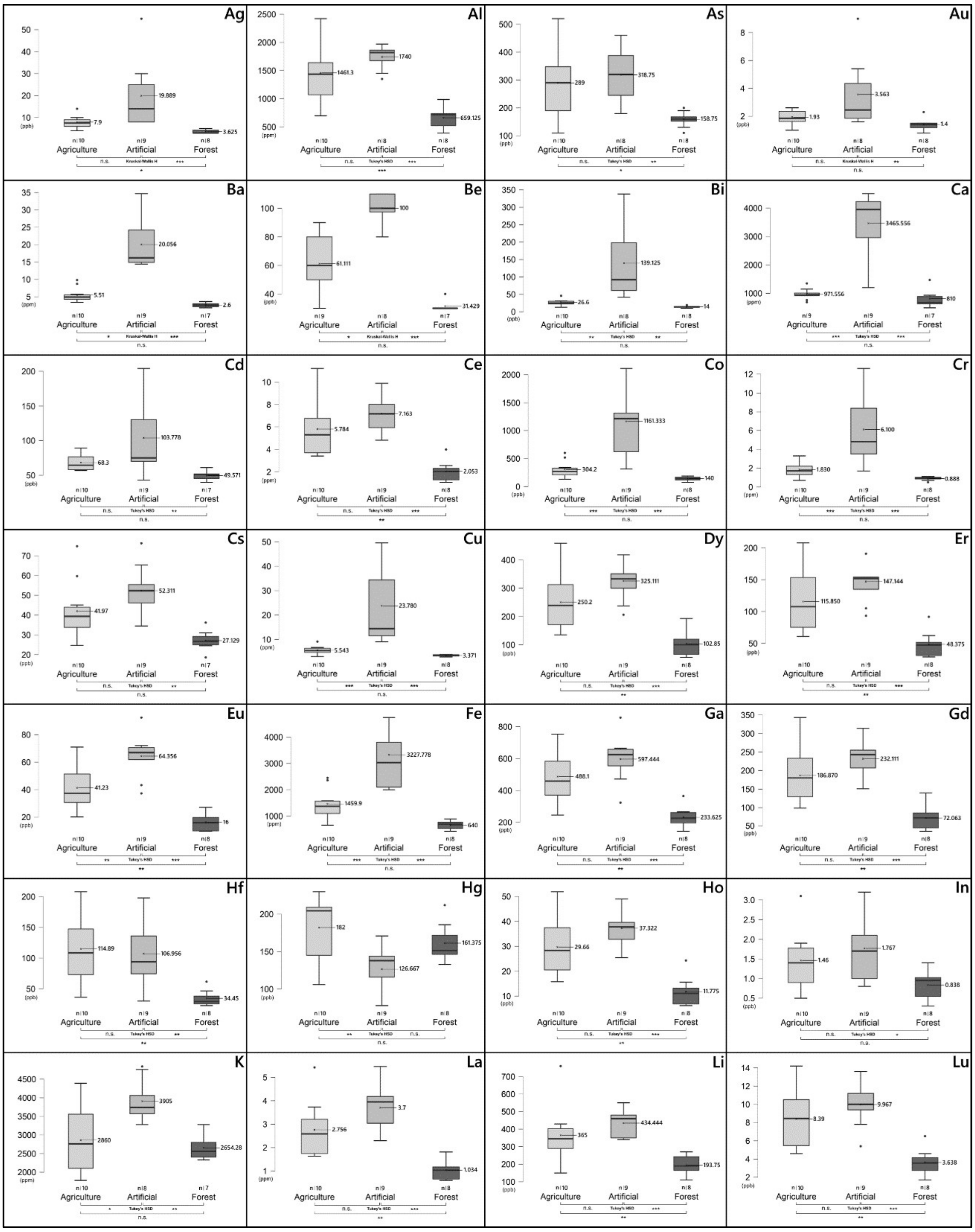

Figure 3. Boxplot diagrams displaying 56 elemental concentration values measured in native lichens from the three major land uses of SMG (Nb and Nd not shown). Mean values of each group are provided at the right side of each box. Units are expressed as ppm (μg/g d.w.) or ppb (ng/g d.w.) Pairwise comparisons of means are shown, and significant differences flagged at $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***) (Tukey's HSD / Kruskal-Wallis H test).

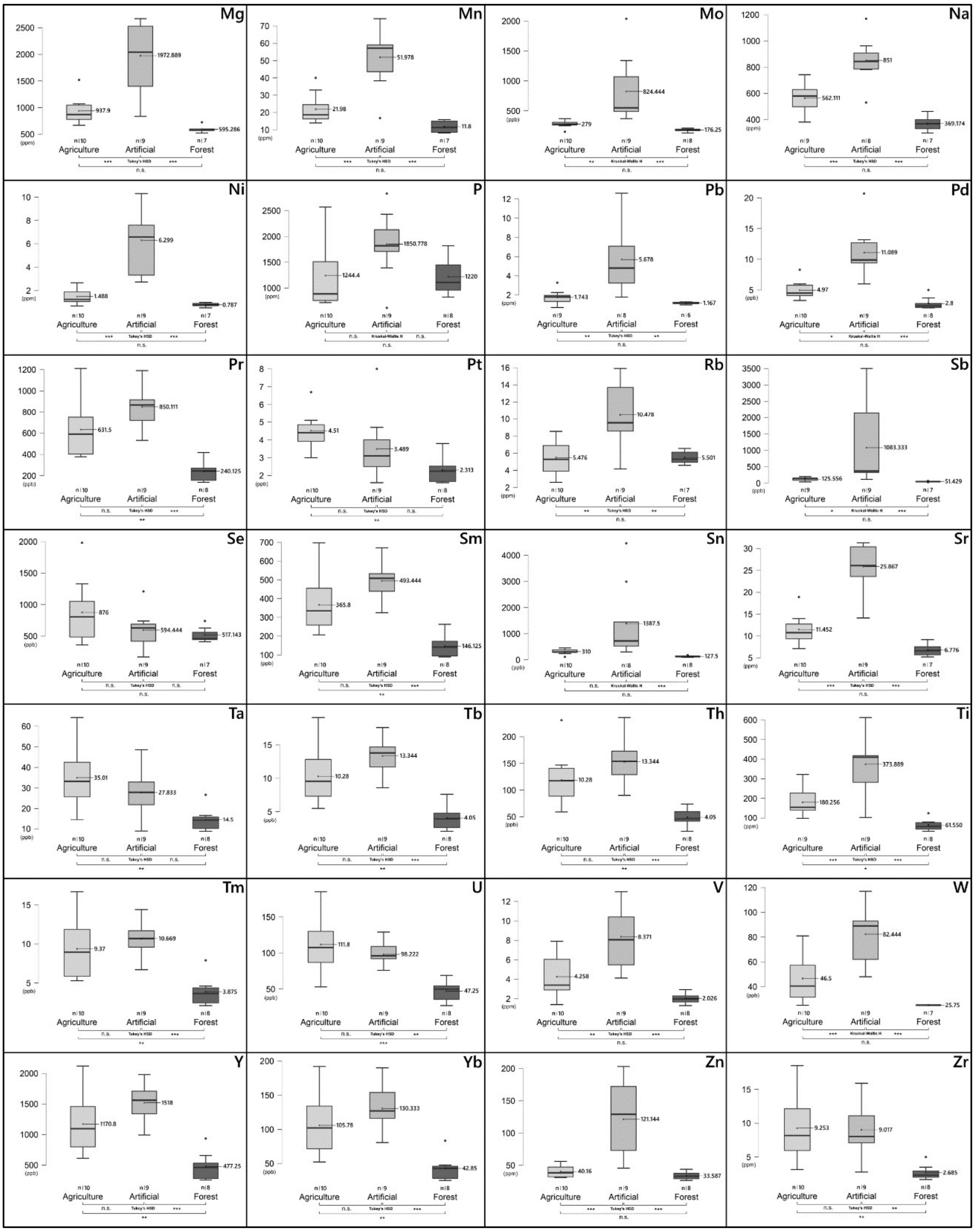

Figure 3. *(continued)*

Some of the highly bioaccumulated elements in lichens from ART land use are heavy metals (Pb, Mo, Mn, V, Zn, Th, Sb, Sn, Co, Ni, Cu, Cr, Ti, Fe), classically defined with reference to environmental pollution and potential toxicity to living organisms (Garty, 2001). In comparison, the mean concentrations of Zn, Cu, Pb, Cr and Ni found in ART lichens were all higher than the mean values recently reported by Port et al. (2018) for urban areas in southern Brazil, in a similar study using *Parmotrema tinctorum* from urban and forest areas. The highest elemental concentrations found by Vieira et al. (2007) on *P. bangii* at a site in eastern Terceira island were likewise attributed to industrial activities (including power-generation units) and to the presence of an international airport and airbase used by US military. The concentrations of Co and Cr were similar, Sb and Zn were lower while Mn, Th and V and others (Ba, Ce, Cs, Eu, Hf, Hg, La, Lu, Rb, Se, Sm) were even higher. These results are especially noteworthy because they reveal how relatively small-scale activities, pertaining to small island populations, can have an environmental impact of considerable magnitude over a pristine background. For instance, the latest annual air quality report for the Azores, based on data from monitoring stations, declares that air quality is good (DRA, 2019b), since the atmospheric concentrations of air pollutants are within legislation limits. However, biomonitoring data revealed an overlooked and significant anthropogenic impact in the context of a pristine and remote oceanic insular location. This highlights the importance of regularly carrying out similar assessments in other seemingly undisturbed and remote insular regions, as these are being promoted for their touristic interest, becoming subjected to intensifying anthropogenic pressure from its massification, adding concern over the preservation of vulnerable island ecosystems. In this regard, this work carried out in SMG island provided a solid contribution of elemental bioaccumulation data across major land uses which is valuable for compilation and comparison with other insular regions facing similar environmental challenges, like neighboring Macaronesia archipelagos in the North Atlantic.

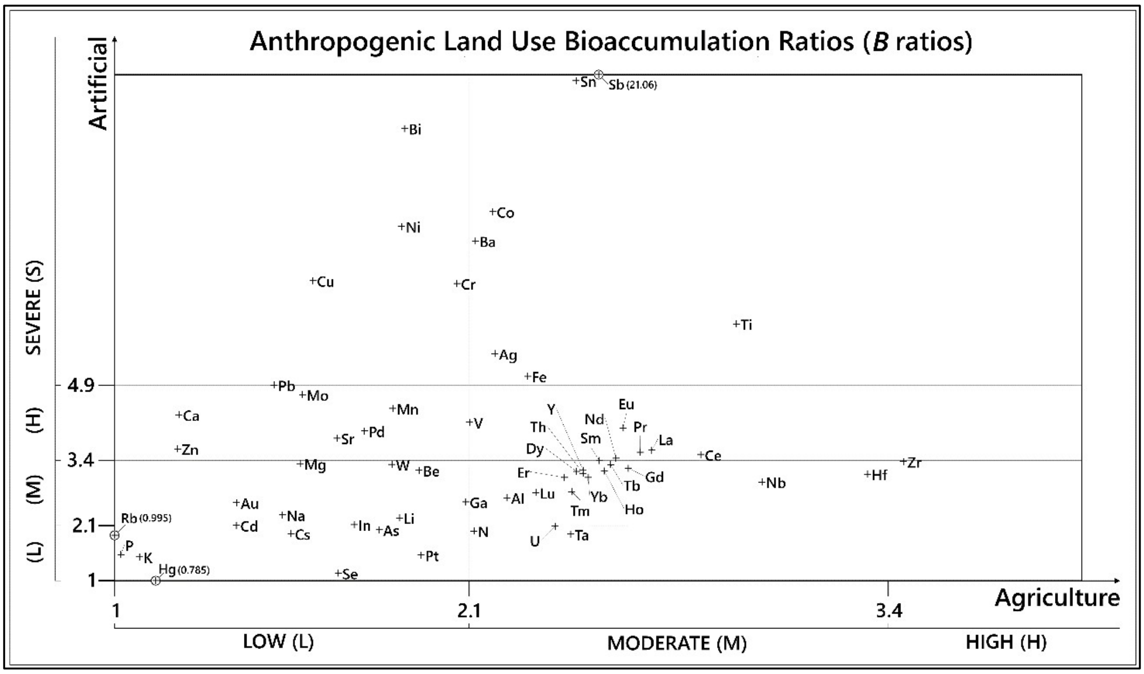

Figure 4. *B* ratios plot comparing the level of elemental bioaccumulation in native lichens from agricultural (x axis) and artificial (y axis) land uses. The ratios thresholds are defined according to the five-class interpretative scale proposed by Cecconi et al. (2019) for bioaccumulation data from native lichens, described as absent (A), low (L), moderate (M), high (H) and severe (S) bioaccumulation.

4. Conclusion

The aim of this study was fulfilled as the biomonitoring approach applied revealed noticeable anthropogenic impact across the land use gradient of São Miguel island. Elemental pollution from human activities linked to agricultural and artificial land uses was reflected by higher bioaccumulation values in native lichens, which were remarkably sensitive even to several rare-earth elements. Lichens from forest sites were noticeably cleaner, providing a mostly exempt pristine background. Overall, agriculture land use reflected a low to moderate impact, while artificial land use, involving urban and industrial activities, reflected a moderate to high impact, including toxic heavy metals. Nitrogen loads were higher on lichens from agricultural sites and their $\delta^{15}N$ isotopic signature was distinguishable from artificial emissions. In face of the greater vulnerability of island ecosystems to anthropogenic impacts, these results provide a data baseline evidencing that relatively small-scale human activities therein require regular monitoring and controlling measures towards the preservation of natural environments.

Credit author statement

Filipe Bernardo: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. Armindo Rodrigues: Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. Cristina Branquinho: Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration. Patricia Garcia: Conceptualization, Formal analysis, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix - Supplementary material

Table S1 . Results of the principal component analysis[(a)].

	PC1	PC2			
Eigenvalue	42.170	10.991			
Variance (%)	64.877	16.908			
Cumulative variance (%)	64.877	81.785			
Variables	Loadings		Variables (cont.)	Loadings (cont.)	
Rb	**0.926**	-0.181	Hf	-0.355	**0.993**
Artificial land use	**0.912**	0.055	Zr	-0.144	**0.992**
P	**0.909**	-0.133	Nb	0.108	**0.916**
Mn	**0.893**	0.217	Tm	0.203	**0.9**
Cr	**0.89**	0.222	Ta	**-0.518**	**0.859**
K	**0.889**	-0.158	Ce	0.287	**0.857**
Mg	**0.887**	0.131	Lu	0.253	**0.853**
Ba	**0.883**	0.184	Ho	0.315	**0.843**
Ca	**0.878**	0.057	Gd	0.33	**0.84**
Cu	**0.875**	0.135	%N	-0.325	**0.837**
Hg	**-0.874**	0.321	Forest land use	0	**-0.802**
Pd	**0.862**	0.258	Er	0.374	**0.795**
Agricultural land use	**-0.859**	**0.546**	Y	0.392	**0.794**
Sr	**0.827**	0.198	La	0.389	**0.794**
Bi	**0.82**	0.177	Dy	0.395	**0.79**
Fe	**0.82**	0.345	Tb	0.401	**0.788**
Co	**0.794**	0.154	Pr	0.403	**0.779**
Pb	**0.792**	0.244	Nd	0.416	**0.77**
Zn	**0.785**	0.1	Th	0.155	**0.77**
Sn	**0.778**	0.129	In	0.169	**0.761**
Na	**0.773**	0.367	Au	0.268	**0.744**
Mo	**0.773**	0.298	Pt	**-0.701**	**0.742**
Sb	**0.765**	0.102	Sm	0.466	**0.733**
Ag	**0.758**	0.348	Al	0.451	**0.725**
Ti	**0.73**	0.428	Yb	0.451	**0.717**
Ni	**0.727**	0.197	Ga	**0.501**	**0.672**
Cd	**0.712**	0.15	%C	-0.465	**-0.639**
$\delta^{15}N$	**0.68**	0.134	Eu	**0.566**	**0.628**
W	**0.638**	0.451	Li	0.463	**0.624**
V	**0.621**	0.394	Cs	0.172	**0.592**
Se	**-0.611**	**0.503**	$\delta^{13}C$	0.222	**0.582**
Be	**0.6**	**0.58**	As	0.264	**0.571**
U	-0.231	**0.998**			

[(a)]Eigenvalues, variance and cumulative variance are shown for the two principal components, along with the variable loadings (sorted by decreasing size) for either component on the obliquely rotated factor solution. Loadings above 0.5 or below -0.5 are highlighted in bold. Considering that both agricultural and urban/industrial activities potentially contribute to increased elemental loads, a correlation between factors was assumed, thus a method for oblique rotation was used, direct oblimin with Kaiser normalization and a Delta of zero; Correlation of 0.327 found between the two components.

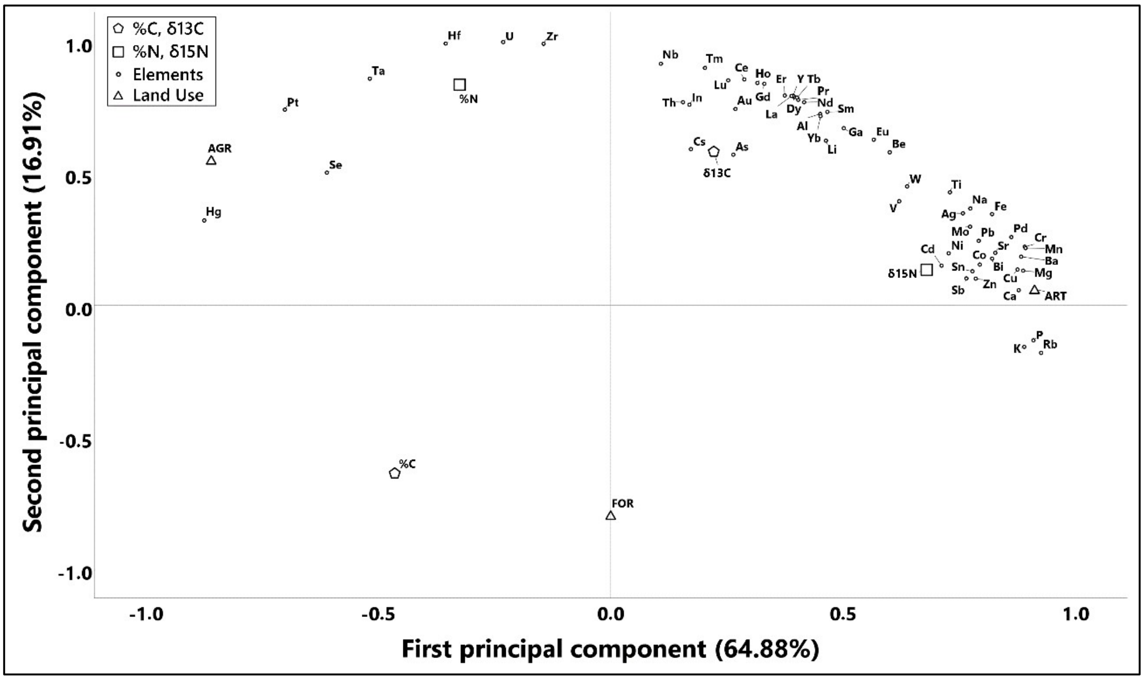


Figure S1. PCA bidimensional component plot in rotated space with 2 principal components accounting for 82.24% of the cumulative variance, distinguishing the elemental profiles between land uses.

Table S2. Descriptive statistics for the elemental concentrations and isotopic ratios of $\delta^{15}N$ and $\delta^{13}C$ quantified in native lichens collected in locations from each of the three main land uses. Highest mean values for each variable are highlighted in bold.

	Units	Land use	n	Max.	Min.	Range	Mean	S.E. Mean	Std. Dev.	Median	M.A.D.
$\delta^{15}N$	‰	AGR	10	-7.38	-15.53	8.14	**-11.16**	0.72	2.29	-11.19	1.49
		ART	9	0.05	-10.78	10.84	-6.23	1.25	3.74	-6.40	3.46
		FOR	8	-6.99	-11.07	4.07	-9.24	0.51	1.45	-9.25	1.27
$\delta^{13}C$	‰	AGR	10	-20.98	-24.31	3.33	-22.33	0.33	1.03	-22.37	0.67
		ART	8	-21.14	-22.58	1.44	-22.06	0.16	0.46	-22.13	0.25
		FOR	7	-22.56	-23.70	1.14	**-23.09**	0.18	0.47	-23.01	0.43
N	%	AGR	10	2.95	1.07	1.87	**2.22**	0.17	0.52	2.21	0.27
		ART	8	2.64	1.67	0.97	2.08	0.11	0.32	2.01	0.17
		FOR	7	1.41	0.65	0.77	1.05	0.11	0.30	1.18	0.24
C	%	AGR	10	43.00	38.72	4.28	41.06	0.47	1.48	41.33	1.10
		ART	9	42.01	37.98	4.03	40.24	0.46	1.38	40.73	0.89
		FOR	8	45.27	42.25	3.01	**43.37**	0.35	0.98	42.98	0.48
Ag	ppb	AGR	10	14.00	4.00	10.00	7.90	0.91	2.89	7.50	1.50
		ART	9	55.00	8.00	47.00	**19.89**	5.20	15.58	14.00	6.00
		FOR	8	5.00	3.00	2.00	3.63	0.32	0.92	3.00	0.00
Al	ppm	AGR	10	2420.00	697.00	1723.00	1461.30	174.24	550.98	1435.00	355.00
		ART	8	1970.00	1350.00	620.00	**1740.00**	78.45	221.88	1820.00	85.00
		FOR	8	986.00	392.00	594.00	659.13	67.76	191.64	716.50	96.50
As	ppb	AGR	10	520.00	110.00	410.00	289.00	39.17	123.87	290.00	90.00
		ART	8	460.00	180.00	280.00	**318.75**	36.57	103.43	320.00	90.00
		FOR	8	200.00	110.00	90.00	158.75	10.25	29.00	160.00	15.00
Au	ppb	AGR	10	2.60	1.00	1.60	1.93	0.16	0.50	1.85	0.30
		ART	8	9.00	1.60	7.40	**3.56**	0.91	2.56	2.45	0.80
		FOR	8	2.30	0.80	1.50	1.40	0.15	0.43	1.40	0.15
Ba	ppm	AGR	10	9.80	3.40	6.40	5.51	0.66	2.08	4.90	0.75
		ART	9	34.70	14.40	20.30	**20.06**	2.36	7.09	16.20	1.80
		FOR	7	3.60	1.80	1.80	2.60	0.24	0.62	2.60	0.40
Be	ppb	AGR	9	90.00	30.00	60.00	61.11	6.76	20.28	60.00	20.00
		ART	8	110.00	80.00	30.00	**100.00**	3.78	10.69	100.00	10.00
		FOR	7	40.00	30.00	10.00	31.43	1.43	3.78	30.00	0.00
Bi	ppb	AGR	10	46.00	13.00	33.00	26.60	2.73	8.63	25.00	3.00
		ART	8	338.00	42.00	296.00	**139.13**	38.54	109.01	92.00	46.50
		FOR	8	19.00	10.00	9.00	14.00	0.93	2.62	14.00	1.00
Ca	ppm	AGR	9	1350.00	687.00	663.00	971.56	65.32	195.95	940.00	70.00
		ART	9	4510.00	1200.00	3310.00	**3465.56**	356.27	1068.80	3950.00	560.00
		FOR	7	1470.00	489.00	981.00	810.00	123.05	325.56	679.00	156.00
Cd	ppb	AGR	10	89.00	57.00	32.00	68.30	3.69	11.65	64.50	7.50
		ART	9	204.00	43.00	161.00	**103.78**	18.12	54.37	75.00	32.00
		FOR	7	61.00	40.00	21.00	49.57	2.60	6.88	51.00	3.00

Ce	ppm	AGR	10	11.20	3.41	7.79	5.78	0.80	2.54	5.29	1.61
		ART	9	9.89	4.81	5.08	**7.16**	0.59	1.77	7.15	1.23
		FOR	8	3.98	1.08	2.90	2.05	0.33	0.94	2.04	0.61
Co	ppb	AGR	10	600.00	133.00	467.00	304.20	46.41	146.77	269.00	63.50
		ART	9	2110.00	314.00	1796.00	**1161.33**	197.28	591.83	1210.00	588.00
		FOR	8	189.00	78.00	111.00	140.00	13.53	38.28	142.50	28.50
Cr	ppm	AGR	10	3.30	0.70	2.60	1.83	0.23	0.74	1.70	0.50
		ART	9	12.60	1.70	10.90	**6.10**	1.13	3.39	4.80	2.40
		FOR	8	1.10	0.50	0.60	0.89	0.07	0.20	0.90	0.15
Cs	ppb	AGR	10	74.80	24.70	50.10	41.97	4.77	15.09	39.35	5.95
		ART	9	76.30	34.50	41.80	**52.31**	4.30	12.90	52.30	6.20
		FOR	7	36.20	18.60	17.60	27.13	2.07	5.47	26.70	2.20
Cu	ppm	AGR	10	9.12	3.02	6.10	5.54	0.54	1.71	5.43	0.96
		ART	9	49.60	9.02	40.58	**23.78**	5.07	15.22	14.40	5.38
		FOR	8	3.85	2.80	1.05	3.43	0.13	0.36	3.50	0.24
Dy	ppb	AGR	10	459.00	135.00	324.00	250.20	32.80	103.73	239.00	76.00
		ART	9	418.00	206.00	212.00	**325.11**	23.26	69.77	333.00	33.00
		FOR	8	193.00	55.10	137.90	102.85	16.00	45.26	100.05	31.35
Er	ppb	AGR	10	208.00	60.80	147.20	115.85	15.58	49.27	107.60	37.85
		ART	9	191.00	93.30	97.70	**147.14**	11.04	33.13	152.00	17.00
		FOR	8	91.70	28.60	63.10	48.38	7.41	20.95	47.35	15.60
Eu	ppb	AGR	10	71.00	20.10	50.90	41.23	5.62	17.78	37.35	11.55
		ART	9	92.50	37.30	55.20	**64.36**	5.40	16.19	67.00	5.10
		FOR	8	27.20	9.40	17.80	16.00	2.32	6.57	15.65	5.95
Fe	ppm	AGR	10	2450.00	639.00	1811.00	1459.90	180.16	569.70	1370.00	255.00
		ART	9	4740.00	2000.00	2740.00	**3227.78**	354.55	1063.66	3030.00	930.00
		FOR	8	887.00	406.00	481.00	640.00	59.71	168.89	677.50	105.50
Ga	ppb	AGR	10	753.00	247.00	506.00	488.10	52.52	166.08	459.50	102.00
		ART	9	857.00	325.00	532.00	**597.44**	48.56	145.68	625.00	41.00
		FOR	8	366.00	143.00	223.00	233.63	23.98	67.82	227.50	36.50
Gd	ppb	AGR	10	343.00	98.70	244.30	186.87	24.18	76.47	180.00	57.00
		ART	9	314.00	151.00	163.00	**232.11**	18.69	56.08	243.00	36.00
		FOR	8	140.00	35.10	104.90	72.06	12.04	34.06	72.60	19.85
Hf	ppb	AGR	10	208.00	36.50	171.50	114.89	17.25	54.55	108.50	38.70
		ART	9	198.00	30.20	167.80	**106.96**	16.21	48.63	94.00	34.00
		FOR	8	61.70	22.80	38.90	34.45	4.78	13.51	29.45	5.70
Hg	ppb	AGR	10	230.00	106.00	124.00	**182.00**	13.93	44.04	204.50	18.00
		ART	9	171.00	78.00	93.00	126.67	10.36	31.07	138.00	21.00
		FOR	8	212.00	133.00	79.00	161.38	9.22	26.07	151.50	12.50
Ho	ppb	AGR	10	51.90	15.80	36.10	29.66	3.72	11.78	28.30	9.10
		ART	9	49.00	25.40	23.60	**37.32**	2.65	7.94	37.80	5.00
		FOR	8	24.30	6.20	18.10	11.78	2.13	6.02	11.10	4.30
In	ppb	AGR	10	3.10	0.50	2.60	1.46	0.23	0.74	1.40	0.45
		ART	9	3.20	0.80	2.40	**1.77**	0.30	0.89	1.70	0.70
		FOR	8	1.40	0.30	1.10	0.84	0.13	0.37	0.95	0.25

		AGR	10	4390.00	1780.00	2610.00	2860.00	289.53	915.57	2760.00	830.00
K	ppm	ART	8	4840.00	3280.00	1560.00	**3905.00**	205.84	582.19	3740.00	215.00
		FOR	7	3280.00	2330.00	950.00	2654.29	126.77	335.40	2560.00	230.00
		AGR	10	5.42	1.63	3.79	2.76	0.38	1.19	2.59	0.79
La	ppm	ART	9	5.46	2.29	3.17	**3.70**	0.34	1.02	3.95	0.51
		FOR	8	1.81	0.60	1.21	1.03	0.15	0.42	1.04	0.36
		AGR	10	760.00	150.00	610.00	365.00	50.65	160.16	345.00	65.00
Li	ppb	ART	9	550.00	340.00	210.00	**434.44**	24.84	74.52	460.00	20.00
		FOR	8	270.00	110.00	160.00	193.75	20.61	58.29	190.00	55.00
		AGR	10	14.20	4.60	9.60	8.39	1.05	3.32	8.45	2.90
Lu	ppb	ART	9	13.60	5.40	8.20	**9.97**	0.79	2.38	10.00	1.20
		FOR	8	6.50	1.70	4.80	3.64	0.53	1.49	3.55	0.85
		AGR	10	1520.00	668.00	852.00	937.90	77.35	244.62	869.50	109.50
Mg	ppm	ART	9	2670.00	836.00	1834.00	**1972.89**	215.70	647.10	2040.00	570.00
		FOR	7	725.00	524.00	201.00	595.29	24.15	63.90	591.00	7.00
		AGR	10	40.00	14.00	26.00	21.98	2.70	8.54	18.70	3.60
Mn	ppm	ART	9	74.20	16.80	57.40	**51.98**	5.75	17.25	57.20	11.50
		FOR	7	15.90	8.20	7.70	11.80	1.29	3.42	11.30	3.10
		AGR	10	370.00	150.00	220.00	279.00	18.10	57.24	280.00	25.00
Mo	ppb	ART	9	2040.00	370.00	1670.00	**824.44**	186.30	558.89	550.00	120.00
		FOR	8	210.00	130.00	80.00	176.25	8.85	25.04	175.00	15.00
		AGR	9	743.00	381.00	362.00	562.11	42.21	126.62	580.00	82.00
Na	ppm	ART	8	1170.00	530.00	640.00	**851.00**	64.16	181.46	843.50	59.50
		FOR	7	463.00	298.00	165.00	369.71	22.59	59.78	368.00	59.00
		AGR	10	2600.00	589.00	2011.00	**1370.70**	200.88	635.25	1185.00	404.00
Nb	ppb	ART	9	2030.00	528.00	1502.00	1345.33	135.93	407.78	1360.00	140.00
		FOR	8	948.00	231.00	717.00	455.75	81.85	231.49	437.50	132.50
		AGR	10	4.44	1.44	3.00	2.38	0.31	0.98	2.20	0.69
Nd	ppm	ART	9	4.22	1.98	2.24	**3.18**	0.26	0.77	3.33	0.57
		FOR	8	1.59	0.50	1.09	0.93	0.13	0.37	0.92	0.29
		AGR	10	2.67	0.68	1.99	1.49	0.20	0.63	1.25	0.43
Ni	ppm	ART	9	10.30	2.74	7.56	**6.30**	0.94	2.83	6.59	3.27
		FOR	7	0.99	0.52	0.47	0.79	0.06	0.17	0.79	0.10
		AGR	10	2570.00	719.00	1851.00	1244.40	211.92	670.15	887.00	160.00
P	ppm	ART	9	2830.00	617.00	2213.00	**1850.78**	209.17	627.50	1820.00	310.00
		FOR	8	1820.00	826.00	994.00	1220.00	130.06	367.86	1110.00	254.00
		AGR	9	3.28	0.71	2.57	1.74	0.25	0.75	1.80	0.46
Pb	ppm	ART	8	12.60	1.79	10.81	**5.68**	1.31	3.71	4.81	2.12
		FOR	6	1.28	0.97	0.31	1.17	0.05	0.12	1.22	0.05
		AGR	10	8.30	3.30	5.00	4.97	0.47	1.47	4.50	0.65
Pd	ppb	ART	9	20.70	6.00	14.70	**11.09**	1.40	4.19	9.90	1.40
		FOR	8	5.00	2.10	2.90	2.80	0.36	1.03	2.40	0.25
		AGR	10	1210.00	378.00	832.00	631.50	85.28	269.69	590.00	191.00
Pr	ppb	ART	9	1190.00	532.00	658.00	**850.11**	71.03	213.09	867.00	148.00
		FOR	8	417.00	138.00	279.00	240.13	33.57	94.95	244.00	78.00

Pt	ppb	AGR	10	6.70	3.00	3.70	**4.51**	0.31	0.98	4.40	0.55
		ART	9	8.00	1.60	6.40	3.49	0.65	1.96	3.10	0.90
		FOR	8	3.80	1.60	2.20	2.31	0.27	0.75	2.25	0.60
Rb	ppm	AGR	10	8.54	2.61	5.93	5.48	0.66	2.08	5.28	1.58
		ART	9	15.90	4.16	11.74	**10.48**	1.32	3.96	9.54	2.79
		FOR	7	6.55	4.58	1.97	5.50	0.29	0.78	5.30	0.70
Sb	ppb	AGR	9	200.00	40.00	160.00	125.56	16.51	49.53	140.00	40.00
		ART	9	3500.00	120.00	3380.00	**1083.33**	412.44	1237.32	370.00	160.00
		FOR	7	70.00	30.00	40.00	51.43	4.59	12.15	50.00	0.00
Se	ppb	AGR	10	1980.00	360.00	1620.00	**876.00**	160.18	506.52	805.00	305.00
		ART	9	1210.00	170.00	1040.00	594.44	98.31	294.92	630.00	160.00
		FOR	7	740.00	410.00	330.00	517.14	45.86	121.34	460.00	30.00
Sm	ppb	AGR	10	697.00	207.00	490.00	365.80	48.07	152.01	334.50	99.00
		ART	9	671.00	325.00	346.00	**493.44**	38.15	114.45	508.00	69.00
		FOR	8	263.00	89.00	174.00	146.13	20.85	58.99	142.00	40.50
Sn	ppb	AGR	10	450.00	110.00	340.00	310.00	30.80	97.41	300.00	45.00
		ART	8	4460.00	300.00	4160.00	**1387.50**	533.39	1508.65	720.00	255.00
		FOR	8	180.00	90.00	90.00	127.50	9.40	26.59	120.00	15.00
Sr	ppm	AGR	10	18.90	7.12	11.78	11.45	1.05	3.32	10.75	1.80
		ART	9	31.30	14.10	17.20	**25.87**	1.83	5.50	26.10	3.10
		FOR	7	9.15	5.20	3.95	6.78	0.53	1.40	6.61	1.13
Ta	ppb	AGR	10	64.20	14.50	49.70	**35.01**	4.52	14.28	33.25	9.40
		ART	9	48.60	8.90	39.70	27.83	3.71	11.12	27.90	6.00
		FOR	8	26.70	8.80	17.90	14.50	2.04	5.76	14.25	3.10
Tb	ppb	AGR	10	19.10	5.50	13.60	10.28	1.34	4.24	9.55	2.85
		ART	9	17.60	8.60	9.00	**13.34**	1.05	3.15	13.80	2.10
		FOR	8	7.60	2.00	5.60	4.05	0.64	1.82	3.90	1.35
Th	ppb	AGR	10	231.00	59.00	172.00	120.20	15.64	49.47	117.50	27.50
		ART	9	236.00	90.00	146.00	**153.00**	15.11	45.34	154.00	25.00
		FOR	8	74.00	22.00	52.00	49.00	6.13	17.33	46.00	10.50
Ti	ppm	AGR	9	322.00	98.30	223.70	180.26	22.61	67.83	155.00	23.00
		ART	9	612.00	103.00	509.00	**373.89**	47.14	141.42	410.00	44.00
		FOR	8	124.00	30.20	93.80	61.55	10.64	30.09	53.50	15.95
Tm	ppb	AGR	10	16.70	5.30	11.40	9.37	1.27	4.00	8.95	3.25
		ART	9	14.40	6.70	7.70	**10.69**	0.80	2.40	10.70	1.10
		FOR	8	7.90	2.10	5.80	3.88	0.66	1.87	3.65	1.05
U	ppb	AGR	10	185.00	53.00	132.00	**111.80**	12.58	39.77	107.50	22.00
		ART	9	129.00	76.00	53.00	98.22	5.49	16.47	96.00	13.00
		FOR	8	69.00	27.00	42.00	47.25	4.87	13.76	50.00	11.00
V	ppm	AGR	10	7.90	1.39	6.51	4.26	0.67	2.12	3.39	1.42
		ART	9	13.00	4.12	8.88	**8.37**	1.06	3.17	8.06	2.58
		FOR	8	2.93	1.29	1.64	2.03	0.20	0.56	1.97	0.37
W	ppb	AGR	10	81.00	25.00	56.00	46.50	6.23	19.70	40.50	12.00
		ART	9	117.00	48.00	69.00	**82.44**	7.64	22.91	89.00	13.00
		FOR	7	25.00	25.00	0.00	25.00	0.00	0.00	25.00	0.00

Y	ppb	AGR	10	2120.00	611.00	1509.00	1170.80	152.30	481.60	1094.50	346.50
		ART	9	1980.00	992.00	988.00	**1518.00**	115.41	346.24	1560.00	220.00
		FOR	8	934.00	256.00	678.00	477.25	81.01	229.14	461.00	180.00
Yb	ppb	AGR	10	192.00	52.30	139.70	105.78	14.16	44.77	102.10	33.25
		ART	9	190.00	80.70	109.30	**130.33**	11.52	34.56	127.00	27.00
		FOR	8	83.40	25.30	58.10	42.85	6.63	18.74	42.90	9.25
Zn	ppm	AGR	10	56.00	31.10	24.90	40.16	2.81	8.88	38.40	6.60
		ART	9	203.00	45.50	157.50	**121.14**	19.05	57.15	129.00	48.90
		FOR	8	43.80	26.60	17.20	33.59	2.11	5.97	33.05	4.70
Zr	ppm	AGR	10	18.50	3.14	15.36	9.25	1.48	4.69	8.18	3.06
		ART	9	15.90	2.78	13.12	9.02	1.25	3.74	8.02	1.72
		FOR	8	5.00	1.67	3.33	2.69	0.39	1.10	2.32	0.47

Table S3. Results for the elemental concentrations and isotopic ratios of $\delta^{15}N$ and $\delta^{13}C$ found in native lichens collected in the vicinity of a quarry compared with the overall maximum value observed for each variable (including other removed outliers). The greater value for each variable is highlighted in bold.

Variables	Quarry value	Maximum observed	Variables	Quarry value	Maximum observed
Ag (ppb)	20.00	**55.00**	Mn (ppm)	**409.00**	74.20
Al (ppm)	**15000.00**	2460.00	Mo (ppb)	1350.00	**2040.00**
As (ppb)	240.00	**1180.00**	Na (ppm)	**3270.00**	1390.00
Au (ppb)	4.7	**27.30**	Nb (ppb)	**4420.00**	2600.00
Ba (ppm)	**53.20**	34.70	Nd (ppm)	**26.50**	4.44
Be (ppb)	**420**	190.00	Ni (ppm)	**53.80**	10.30
Bi (ppb)	65.00	**894.00**	P (ppm)	2460.00	**2830.00**
Ca (ppm)	**12300.00**	4510.00	Pb (ppm)	3.80	**27.20**
Cd (ppb)	103.00	**204.00**	Pd (ppb)	**49.40**	20.70
Ce (ppm)	**53.5**	11.20	Pr (ppb)	**6670.00**	1210.00
Co (ppb)	**14800.00**	2110.00	Pt (ppb)	**6.2**	8.00
Cr (ppb)	**68.80**	12.60	Rb (ppm)	**17.10**	15.90
Cs (ppb)	**149.00**	76.30	Sb (ppb)	90.00	**3500.00**
Cu (ppm)	33.20	**49.60**	Se (ppb)	200.00	**1980.00**
Dy (ppb)	**2510.00**	459.00	Sm (ppb)	**4280.00**	697.00
Er (ppb)	**1040.00**	208.00	Sn (ppb)	**1160.00**	11900.00
Eu (ppb)	**578.00**	92.50	Sr (ppm)	**123.00**	31.30
Fe (ppm)	**32600.00**	4740.00	Ta (ppb)	54.90	**64.20**
Ga (ppb)	**5040.00**	857.00	Tb (ppb)	**109.00**	19.10
Gd (ppb)	**1980.00**	343.00	Th (ppb)	**1940.00**	236.00
Hf (ppb)	**690.00**	208.00	Ti (ppm)	**4170.00**	612.00
Hg (ppb)	47.00	**230.00**	Tm (ppb)	**71.5**	16.70
Ho (ppb)	**278.00**	51.90	U (ppb)	**301.00**	185.00
In (ppb)	**11.5**	3.20	V (ppm)	**85.30**	13.00
K (ppm)	3580.00	**4840.00**	W (ppb)	**150.00**	117.00
La (ppm)	**25.90**	5.46	Y (ppb)	**11000.00**	2120.00
Li (ppb)	**2030**	760.00	Yb (ppb)	**794.00**	192.00
Lu (ppb)	**57.4**	14.20	Zn (ppm)	95.20	**203.00**
Mg (ppm)	**12300.00**	2670.00	Zr (ppb)	**48.50**	18.50
N (%)	0.419	**2.947**	C (%)	9.559	**45.268**
$\delta^{15}N$ (‰)	-9.592	**0.054**	$\delta^{13}C$ (‰)	-25.374	**-20.980**

Table S4. Values of blanks and duplicates as well as percentages of recovery of the certified reference material to check analytical accuracy of the quantification of elemental concentrations in native lichens, presented in tables S2 and S3[a].

Elements	Units	Blank	Sample 1	Duplicate Sample 1	Sample 2	Duplicate Sample 2	Percentages of recovery
Ag	ppb	< 3	6	6	32	28	88.89%
Al	ppm	< 4	628	682	2080	1870	88.00%
As	ppb	< 10	160	170	1250	1110	90.77%
Au	ppb	0.6	5.5	3.7	9.5	8.6	173.91%
Ba	ppm	< 0.1	4	3.9	37.4	32	100.00%
Be	ppb	< 30	30	< 30	100	100	N/A
Bi	ppb	< 2	20	22	938	849	135.00%
Ca	ppm	< 25	693	764	4110	4010	97.42%
Cd	ppb	< 6	39	41	135	115	100.86%
Ce	ppm	< 0.015	1.99	2.1	8.65	7.36	88.05%
Co	ppb	< 4	136	141	1350	1180	73.77%
Cr	ppm	< 0.1	1.1	1.1	13.7	11.4	82.64%
Cs	ppb	< 0.2	17.6	22.1	53	56.2	49.42%
Cu	ppm	< 0.05	3.55	3.97	51.5	47.8	96.79%
Dy	ppb	< 0.5	99.5	113	352	314	N/A
Er	ppb	< 0.4	45.7	45.7	161	144	N/A
Eu	ppb	< 0.2	18.7	21.3	72.8	63.3	N/A
Fe	ppm	< 3	653	689	5100	4380	86.96%
Ga	ppb	< 4	207	207	207	207	73.50%
Gd	ppb	< 0.4	68.4	68.4	68.4	68.4	N/A
Hf	ppb	0.7	32.2	35.3	155	120	57.61%
Hg	ppb	< 2	211	219	148	128	97.74%
Ho	ppb	< 0.2	12.8	12	41	34.5	N/A
In	ppb	< 0.2	0.4	1.1	0.5	1.4	N/A
K	ppm	< 10	2220	2330	4740	4950	86.67%
La	ppm	< 0.01	0.93	1	4.23	3.67	90.91%
Li	ppb	< 10	160	170	580	510	69.64%
Lu	ppb	< 0.5	3.2	3.8	10.2	8.6	N/A
Mg	ppm	< 2	614	641	2700	2520	83.21%
Mn	ppm	< 0.1	10.8	11.2	73.6	63.9	76.76%
Mo	ppb	< 10	160	170	2110	1960	90.00%
Na	ppm	< 5	663	708	893	888	73.02%
Nb	ppb	< 2	390	420	1760	1550	35.00%
Nd	ppm	< 0.005	0.875	0.979	3.6	3.06	N/A
Ni	ppm	< 0.05	0.73	0.72	6.97	6.21	91.72%
P	ppm	< 50	783	847	2430	2440	97.20%
Pb	ppm	< 0.05	2.12	2.2	10.3	8.55	107.78%
Pd	ppb	< 0.2	3.3	2.7	21.5	19.9	N/A
Pr	ppb	< 1	227	239	930	805	N/A

Pt	ppb	< 0.2	1.9	2	7.9	8.1	N/A
Rb	ppm	< 0.01	3.73	3.85	11.1	11.2	73.94%
Sb	ppb	< 10	170	210	3700	3310	100.00%
Se	ppb	< 10	510	200	580	800	70.71%
Sm	ppb	< 1	149	152	551	466	N/A
Sn	ppb	< 50	230	220	12600	11200	100.00%
Sr	ppm	< 0.04	7.62	8.37	32	30	82.79%
Ta	ppb	0.6	15.5	15.8	40.2	30.4	N/A
Tb	ppb	< 0.2	3.9	4.3	14.7	12.9	N/A
Th	ppb	< 2	52	56	258	214	87.38%
Ti	ppm	< 0.15	72.1	75	437	383	89.33%
Tm	ppb	< 0.1	3.7	4	11.7	10.6	N/A
U	ppb	< 1	41	45	112	89	64.12%
V	ppm	< 0.01	1.71	1.8	8.62	7.49	99.76%
W	ppb	< 25	< 25	< 25	110	94	N/A
Y	ppb	< 2	477	509	1640	1480	87.94%
Yb	ppb	< 0.4	39.7	46.1	147	130	N/A
Zn	ppm	< 0.4	36.9	39.5	176	170	100.53%
Zr	ppm	< 0.02	2.68	2.79	13.1	10.8	86.05%

[(a)]Some of the samples chosen for duplicated analysis had outlier values for some elements which were removed from descriptive statistics and thus can be outside the range reported in table S2. Elements lacking % of recovery (N/A) have no certified values in the certificate of analysis provided by the supplier of the reference materials.

Table S5. Mean values of *B* ratios and corresponding 95% confidence intervals pertaining to Figure 4 of the main manuscript.

ARTIFICIAL				AGRICULTURE			
Element	***B* ratios**	**95% CI (± mean)**	**Bioaccumulation class**	**Element**	***B* ratios**	**95% CI (± mean)**	**Bioaccumulation class**
Hg	0.785	0.119	ABSENCE	Rb	0.995	0.222	ABSENCE
Se	1.149	0.351	LOW	P	1.020	0.323	LOW
K	1.471	0.142		K	1.078	0.203	
Pt	1.509	0.522		Hg	1.128	0.160	
P	1.517	0.317		Zn	1.196	0.155	
Rb	1.905	0.444		Ca	1.199	0.149	
Ta	1.920	0.472		Cd	1.378	0.138	
Cs	1.928	0.293		Au	1.379	0.211	
N	1.981	0.195		Pb	1.494	0.395	
As	2.008	0.422		Na	1.520	0.211	
U	2.079	0.215		Cs	1.547	0.327	
Cd	2.093	0.676		Mg	1.576	0.242	
In	2.109	0.655	MODERATE	Mo	1.583	0.191	
Li	2.242	0.237		Cu	1.615	0.292	
Na	2.302	0.318		Sr	1.690	0.288	
Au	2.545	1.185		Se	1.694	0.576	
Ga	2.557	0.384		In	1.743	0.520	
Al	2.640	0.218		Pd	1.775	0.309	
Lu	2.740	0.402		As	1.820	0.459	
Tm	2.758	0.382		W	1.860	0.463	
Nb	2.952	0.551		Mn	1.863	0.425	

Yb	3.042	0.497		Li	1.884	0.486	
Er	3.042	0.422		Ni	1.890	0.470	
Hf	3.105	0.870		Bi	1.900	0.362	
Th	3.122	0.570		Be	1.944	0.397	
Dy	3.161	0.418		Pt	1.950	0.250	
Ho	3.170	0.415		Cr	2.062	0.488	
Y	3.181	0.447		Ga	2.089	0.418	
Be	3.182	0.220		V	2.101	0.614	
Gd	3.221	0.479		N	2.114	0.292	
Tb	3.295	0.479		Ba	2.119	0.471	
W	3.298	0.565		Co	2.173	0.616	
Mg	3.314	0.670		Ag	2.179	0.468	
Zr	3.358	0.857		Al	2.217	0.492	
Sm	3.377	0.482		Fe	2.281	0.523	
Nd	3.422	0.508	HIGH	Lu	2.307	0.536	
Ce	3.490	0.530		U	2.366	0.495	
Pr	3.540	0.547		Er	2.395	0.599	
La	3.579	0.605		Ta	2.414	0.579	
Zn	3.607	1.048		Tm	2.418	0.607	
Sr	3.818	0.500		Sn	2.431	0.449	
Pd	3.960	0.921		Dy	2.433	0.593	
Eu	4.022	0.623		Sb	2.441	0.593	
V	4.131	0.963		Th	2.453	0.594	MODERATE
Ca	4.278	0.813		Y	2.453	0.593	
Mn	4.405	0.900		Yb	2.469	0.614	
Mo	4.678	1.953		Sm	2.503	0.612	
Pb	4.866	2.062		Ho	2.519	0.588	
Fe	5.043	1.024		Tb	2.538	0.615	
Ag	5.487	2.648		Nd	2.555	0.616	
Ti	6.075	1.415		Eu	2.577	0.653	
Cr	6.873	2.354		Gd	2.593	0.624	
Cu	6.930	2.732		Pr	2.630	0.660	
Ba	7.714	1.680	SEVERE	La	2.666	0.679	
Ni	8.002	2.214		Ce	2.818	0.728	
Co	8.295	2.604		Ti	2.929	0.679	
Bi	9.938	5.047		Nb	3.008	0.820	
Sn	10.882	7.670		Hf	3.335	0.931	
Sb	21.065	14.820		Zr	3.446	1.027	HIGH

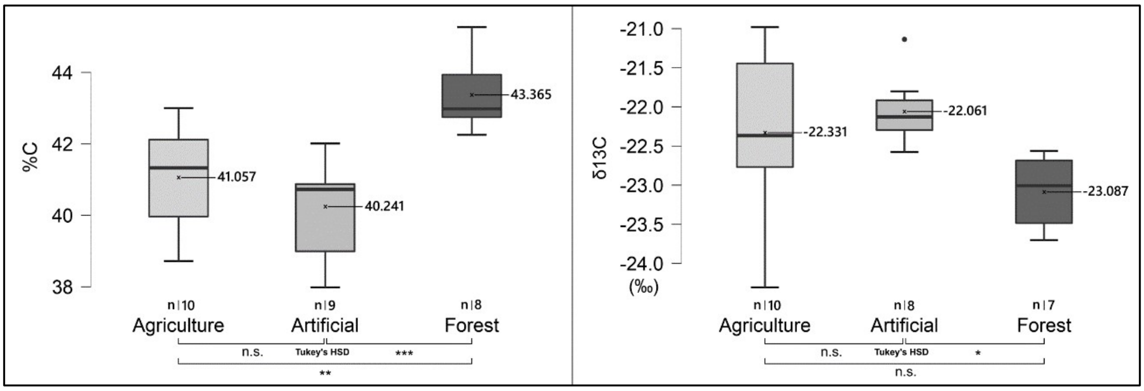


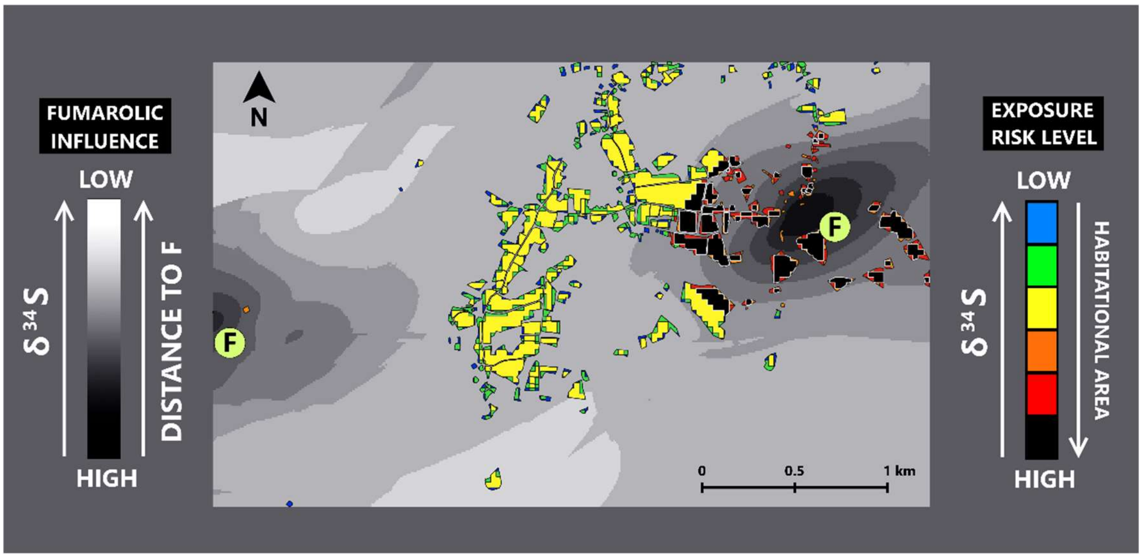
Figure S2. Boxplots displaying carbon concentration (C, %) and isotopic composition ($\delta^{13}C$, ‰) values measured in native lichens[(a)] from the three major land uses of SMG. Mean values of each group are provided at the right side of each box. Pairwise comparisons of means are shown, and significant differences flagged at $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***) (Tukey's HSD).

[(a)]**Analytical procedure** : $\delta^{13}C$ was determined on dried macerated lichen samples by continuous flow isotope-ratio mass spectrophotometry (CF-IRMS) (Preston and Owens, 1983) for vegetation matrices (LIE-SIIAF, 2020). More specifically, analyses of $\delta^{13}C$ were carried out on a Sercon Hydra 20-22 (Sercon, UK) stable isotope ratio mass spectrometer, coupled to a EuroEA (EuroVector Italy) elemental analyzer for online sample preparation by Dumas-combustion. Carbon delta (δ) was calculated by $\delta = [(R_{sample} / R_{standard}) \times 1000]$, expressed also in ‰ units, where R is the ratio of $^{13}C/^{12}C$ stable isotopes. Analytical quality control for $\delta^{13}C$ was ensured by the analysis of two different primary reference materials: IAEA-CH7 with $\delta^{13}C_{VPDB}$ = -2.151 ± 0.05‰; Glucose BCR with $\delta^{13}C_{VPDB}$ = -10.76 ± 0.04‰. A secondary reference material WFS OAS (with $\delta^{13}C_{VPDB}$ = -27.43 ± 0.03‰) was checked against these primary reference materials (Coleman and Meier-Augenstein, 2014). $\delta^{13}C_{VPDB}$ refers to "Vienna Pee Dee Belemnite". Measurement accuracy was checked by the analysis of 9 replicates of the secondary isotopic reference material WFS OAS, interspersed amongst the sample batch, from which analytical variance was calculated with std deviation of 0.08‰. The major mass signals of C were used to calculate total C abundance using WFS OAS as elemental composition reference material with 39.53% C (LIE-SIIAF, 2020). The references cited are in the references list.

CHAPTER 3

Lichens as biomonitors of volcanic hydrothermal emissions

3.1. Spatially modelling the risk areas of chronic exposure to hydrothermal volcanic emissions using lichens



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Spatially modelling the risk areas of chronic exposure to hydrothermal volcanic emissions using lichens

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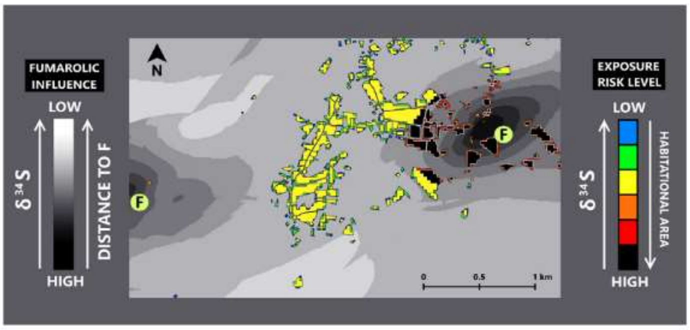
HIGHLIGHTS

- $\delta^{34}S$ in lichens is a time-integrated tracer of airborne hydrothermal emissions.
- The model efficiently assessed chronic outdoor exposure to volcanic air pollution.
- Habitational risk areas are shown in a high spatial resolution map integrating time.
- The methodologic approach is replicable in other volcanic areas of the world.
- The developed tool is useful to uphold health safety measures for the volcanic areas.

GRAPHICAL ABSTRACT



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S isotopic ratio

ABSTRACT

Human populations living in volcanically active areas are chronically exposed to volcanogenic air pollution, potentially contributing to long-term adverse health effects. However, mapping chronic exposure is difficult due to low spatial resolution of monitoring data on air pollutants and the need for time integration. To overcome these problems, lichens were tested as ecological indicators of hydrothermal volcanic air pollution, considering their bioaccumulation capacity over time, by transplanting them from a reference area to several sites ($n = 39$) in a volcanic area. The test was developed at Furnas volcano (Azores, Portugal). A stratified sampling design was followed using previous measurements of soil CO_2 flux at ground level and the distance to the main fumarolic fields. After 6 months of exposure, lichen transplants were analyzed for S isotopic ratio ($\delta^{34}S$), which strongly related with the distance to fumarolic fields on a logarithmic regression, serving as an appropriate hydrothermal exposure biomarker. Considering kriging interpolated $\delta^{34}S$ values as tracer of airborne hydrothermal emissions and habitational areas as proxy of ongoing human presence, a map was built relating both information per area unit to spatially model risk areas. It was estimated that 26% of habitational areas in the study area stand at high or very high risk of outdoors chronic exposure to airborne hydrothermal emissions. This methodologic approach to produce chronic exposure risk maps is applicable to other volcanically active and inhabited areas of the world, with time-integration and high spatial resolution, contributing in this way for spatially focusing future human health assessments.

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Abstract

Human populations living in volcanically active areas are chronically exposed to volcanogenic air pollution, potentially contributing to long-term adverse health effects. However, mapping chronic exposure is difficult due to low spatial resolution of monitoring data on air pollutants and the need for time integration. To overcome these problems, lichens were tested as ecological indicators of hydrothermal volcanic air pollution, considering their bioaccumulation capacity over time, by transplanting them from a reference area to several sites (n = 39) in a volcanic area. The test was developed at Furnas volcano (Azores, Portugal). A stratified sampling design was followed using previous measurements of soil CO_2 flux at ground level and the distance to the main fumarolic fields. After 6 months of exposure, lichen transplants were analyzed for S isotopic ratio ($\delta^{34}S$), which strongly related with the distance to fumarolic fields on a logarithmic regression, serving as an appropriate hydrothermal exposure biomarker. Considering kriging interpolated $\delta^{34}S$ values as tracer of airborne hydrothermal emissions and habitational areas as proxy of ongoing human presence, a map was built relating both information per area unit to spatially model risk areas. It was estimated that 26% of habitational areas in the study area stand at high or very high risk of outdoors chronic exposure to airborne hydrothermal emissions. This methodologic approach to produce chronic exposure risk maps is applicable to other volcanically active and inhabited areas of the world, with time-integration and high spatial resolution, contributing in this way for spatially focusing future human health assessments.

Highlights:

- $\delta^{34}S$ in lichens is a time-integrated tracer of airborne hydrothermal emissions.
- The model efficiently assessed chronic outdoor exposure to volcanic air pollution.
- Habitational risk areas are shown in a high spatial resolution map integrating time.
- The methodologic approach is replicable in other volcanic areas of the world.
- The developed tool is useful to uphold health safety measures for the volcanic areas.

Keywords: Air pollution; Volcanism; Chronic exposure; Lichens; Biomonitoring; S isotopic ratio.

1. Introduction

It was recently estimated that around 10% of the world's total human populations inhabit volcanically active areas (Small and Naumann, 2001). The spatial coincidence of volcanoes and populations is currently increasing as dense and rapidly growing populations occupy many volcanically active regions (Small and Naumann, 2001). The volcanic areas are subjected not only to potentially devastating, short-lived eruption episodes but also to chronic exposure to volcanogenic air pollution through degassing manifestations, such as fumaroles, occurring both during states of volcanic eruption and quiescence (Longo et al., 2010). Volcanic emissions contain hazardous gases to humans, aerosols and particles, including CO, CO_2, SO_2, H_2S, HCl, HF, radon and heavy metals like Pb, As and Hg (Hansell and Oppenheimer, 2004; Hansell et al., 2006; Williams-Jones and Rymer, 2015), which can cause adverse health effects, particularly cardiorespiratory conditions (Hansell and Oppenheimer, 2004; Curtis et al., 2006), following not only acute overexposures but also repeated or chronic exposures to lower atmospheric concentrations (Williams-Jones and Rymer, 2015).

On Furnas village, located inside the caldera of the quiescent Furnas volcano, São Miguel Island, Azores, previous research established weight of evidence concerning adverse health effects on Furnas inhabitants, particularly chronically induced conditions on the respiratory tract, including increased incidence of respiratory system related cancers (Amaral et al., 2006), increased incidence of chronic bronchitis (Amaral and Rodrigues, 2007) and increased prevalence of restrictive defects and chronic obstructive pulmonary disease (COPD) (Linhares et al., 2015). In addition, other harmful effects are recognized at genotoxic and cytotoxic levels, such as chromosome damage on oral epithelial cells (Linhares et al., 2018) and increased frequency of micronucleated cells and cells with other nuclear anomalies (Rodrigues et al., 2012).

It is challenging to assess chronic exposure to air pollution in outdoor conditions, such as that of human populations living in volcanic environments. Since the main route of human exposure is through inhalation of the air mixture, the time-integrated dispersal patterns of airborne pollutants on outdoor conditions must be accounted for. Once diluted in the atmosphere, air pollutants dissipate over distance and might occur in very low concentrations, which are difficult to measure (Davies and Notcutt, 1996; Augusto et al., 2004). Furthermore, their dispersal through dry and wet deposition is subjected to variable weather conditions, such as rainfall, wind strength and direction (Davies and Notcutt, 1996; Conti and Cecchetti, 2001). Data provided by conventional sampling procedures, such as those used in air quality monitoring stations, lacks the spatial representativeness required for determining patterns of chronic exposure to air pollutants (Augusto et al., 2012). The cost of the equipment used in these stations allows for only a few sites to be monitored (Garty, 2001; Augusto et al., 2012). These analytical and logistical limitations of conventional monitoring stations can be lessened by cost-saving complementation with ecological indicators (Rossbach et al., 1999; Garty, 2001; Augusto et al., 2004).

Lichens are amongst the best and most widely used ecological indicators of air pollution (Conti and Cecchetti, 2001; Williamson et al., 2008), including volcanic gas emissions (Bargagli and Barghigiani, 1991; Davies and Notcutt, 1996; Grasso et al., 1999). As poikilohydric organisms, lichens lack complex mechanisms for water content and gas exchange regulation (such as cuticle and stomata in higher plants), depending almost exclusively on atmospheric input for mineral nutrition and water. By continually assimilating chemical elements available in the atmosphere, lichens accurately reflect, over long periods of time, the elemental composition of the surrounding atmosphere in their biological matrix, therefore revealing the time-integrated wet and dry deposition of air pollutants, providing cost-effective tools for mapping spatial and temporal patterns of air pollution (Grasso et al., 1999; Garty, 2001; Batts et al., 2004; Williamson et al., 2008; Bačkor and Loppi, 2009; Barros et al., 2015). Reproducing the long-term exposure to complex low-level mixtures of air pollutants is critical for relating air pollution data with chronic effects on human health (Wolterbeek, 2002; Ribeiro et al., 2010).

The hypothesis of this study is that lichens are suitable biomonitoring tools to spatially model, with time integration, the risk areas of outdoors chronic exposure to hydrothermal air pollution. To do so, a fruticose lichen species was transplanted and exposed for 6 months at Furnas village, which served as the study area. S isotopic ratio ($\delta^{34}S$) was measured on the lichen matrices and related with *in-situ* measurements of soil CO_2 flux and distance to the Furnas volcano caldera fumarolic fields, to assess its aptness as tracer to spatially predict the long-term dispersal of airborne hydrothermal emissions, in outdoor conditions, and map the risk areas of chronic exposure.

2. Materials and methods

2.1. Study area

The locations of the study area and reference site are provided in Fig. 1. Furnas volcano is plentiful on degassing manifestations (Viveiros et al., 2009), including soil diffuse degassing emissions (968 $t.d^{-1}$), occurring throughout the volcano's caldera, in a mostly unnoted process (Viveiros et al., 2010), as well as more focalized and noticeable surface manifestations of hydrothermal activity, such as fumarolic discharges (50 $t.d^{-1}$) (Pedone et al., 2015), thermal and cold CO_2 springs (12 $t.d^{-1}$) (Cruz et al., 1999), within a relative distribution like other volcanic-geothermal areas of the world (Viveiros et al., 2012). Both degassing avenues represent chronic exposures for Furnas village population. On one hand, in Furnas village, 58% of houses are built on top of hydrothermal soil diffuse degassing ground (Viveiros et al., 2010), potentiating indoor CO_2 and radioactive gas radon (^{222}Rn) accumulation in hazardous concentrations for dwellers, specially under poor ventilation conditions (Baxter et al., 1999; Viveiros et al., 2009; Silva et al., 2014; Silva et al., 2015; Linhares et al., 2018). On the other hand, each of the main fumarolic fields (Fig. 2) contain several moderate to large actively degassing fumaroles within an area of several hundred m^2 (Pedone et al., 2015), presenting potential health hazards for villagers and tourists (Heggie, 2009). The composition of the fumaroles gaseous discharges consists mostly of water vapor and CO_2, followed by H_2S, H_2, N_2, O_2, CH_4, CO and Ar (Ferreira and Oskarsson, 1999; Viveiros et al., 2009; Caliro et al., 2015; Pedone et al., 2015), which can carry along volcanogenic aerosols and particulate matter.

2.2. Sampling design

The sampling design was based on the different CO_2 concentration classes from soil diffuse degassing (g m^{-2} d^{-1}) reported by Viveiros et al., 2010 on their distribution map of soil CO_2 flux for Furnas caldera (Fig. 2), to which the CO_2 diffuse degassing structures at Furnas volcano are mostly associated (Viveiros et al., 2010). Specifically, 50 different locations were selected in the study area, distributed over space, stratifying by potential exposure to volcanogenic air pollution. The potential exposure levels to volcanogenic air pollution were based on the 10 classes of soil CO_2 flux degassing gradient defined by Viveiros et al., 2010 for Furnas caldera (Fig. 2). A balanced number of four locations were chosen within each class of the soil CO_2 flux gradient, except for the two highest flux classes, closer to the three main fumarolic fields, each including seven locations (Fig. 2). Locations were chosen ensuring a minimum distance of 20 m to the nearest transplant and to cover homogeneously the study area. Transplant locations were adjusted in the field to the nearest suitable location. The geocoordinates of the transplants' locations were recorded and their linear distance to each fumarolic field was determined. Overall, the average distance between transplants was 174 m (minimum = 24 m; maximum = 471 m). The exposure period lasted from July 2017 to January 2018. Three samples were re-transplanted on the reference site during the same period, serving as transplant-control and as a reference to obtain background values assuredly not influenced by exposure to hydrothermal emissions. After the exposure period, 39 lichen transplants were retrieved for analysis, while the remaining 11 were lost due to vandalism or extreme degradation, including the 4 transplants placed around Ribeira dos Tambores fumarolic field (Fig. 2).

2.3. Lichen transplants

Usnea rubicunda was selected as the biomonitor lichen species for transplantation. Besides easily identifiable, easy to collect, handle and transplant, this lichen species occurs natively in the vicinity of Furnas fumarolic fields, providing simultaneously a degree of tolerance to long-term exposure to hydrothermal air pollution and, due to its shrubby fruticose growth-form, greater interaction with the surrounding air and enhanced particle interception (Branquinho et al., 2008). Lichen transplantation involves the relocation of specimens naturally occurring in a relatively unimpacted environment to the area of interest, subject to air pollution, for a fixed exposure period (Mikhailova, 2002; Williamson et al., 2008). This strategy has practical advantages over *in-situ* sampling of pre-existing lichens, such as repeatability, greater flexibility in site selection and spatial distribution, well-defined exposure time and ensuring exposure free condition at its beginning (Caggiano et al., 2015). The *U. rubicunda* lichens used for transplantation to the study area were collected from *Cryptomeria japonica* trunks in the reference site, corresponding to a forest reserve located outside the Furnas volcano caldera (Fig. 1). Both the study area (approx. 3.5 km) and the reference site (approx. 2.6 km) are close to the ocean. The transplanted lichen thalli were lodged in transplant bags of 40 cm in length, consisting of a porous stretchable nylon net, allowing free airflow. On the study area, the transplant bags were placed at around 1.5 m of height from the ground, approximately the human breathing height, on available supports like tree trunks and posts. To promote unconstrained ambient exposure, it was sought that each transplant support was clear from surrounding obstacles at transplant height, such as dense tree foliage, potentially limiting sunlight, air circulation and rainfall. Nevertheless, attaining completely open field placement or avoiding closeness to a nearby road was unachievable for some transplants, though traffic intensity is very low in Furnas village. The reference samples, kept on the reference site, were lodged in identical transplant bags placed at a similar height, accounting for the same experimental handling as Furnas transplants.

2.4. Sample processing

The 39 exposed transplants and 3 reference samples were simultaneously retrieved and, shortly afterwards, processed on the laboratory. The totality of lichen material contained within a single transplant bag was processed independently as one sample, excluding visibly moldy or damaged lichen portions which were removed, usually corresponding to innermost thalli precipitated on the bottom of the transplant bag due to strong wind agitation, therefore lacking sunlight access and air drying. The selected lichen material was oven dried at 40 °C for 72 h. No preliminary water cleansing was made to prevent the washing away of a significant elemental load. Around 10% of the total weight was lost after oven drying.

Homogenization of dried lichen material into powder was done by removing tree bark leftovers, cutting with stainless-steel scissors and manual grinding with mortar and pestle.

2.5. Laboratory analysis

Stable isotopic values of S ($\delta^{34}S$) were determined on a portion of the homogenized lichen material of each transplant by isotope-ratio mass spectrophotometry (IRMS) for vegetation matrices (LIE-SIIAF, 2018). More specifically, analyses of $\delta^{34}S$ were carried out on a continuous flow IRMS (CF-IRMS) (Preston and Owens, 1983) on an Isoprime (GV, UK) stable isotope ratio mass spectrometer, coupled to a EuroEA (EuroVector Italy) elemental analyser for online sample preparation by Dumas combustion for quantification of C, N, S, H and O. Isotopic S results refer to troilite from Viena Canyon Diablo meteorite, expressed in δ notation (per mil, ‰), corresponding to the ratio of the two most common stable S isotopes, ^{34}S and ^{32}S, measured in the samples, against the same ratio measured in the reference standard, accordingly with the following formula (Reuss et al., 1987; Barros et al., 2015).

$$\delta^{34}\mathrm{S} = \left[\frac{^{34}S/^{32}S\ \mathrm{sample}}{^{34}S/^{32}S\ \mathrm{standard}}\right] \times 1000$$

Analytical quality control for S isotopic composition was ensured by analysis of two certified primary reference materials of the International Atomic Energy Agency (IAEA) - IAEA-S1 and IAEA-NBS127, as well as a secondary reference material (internal standard) (Coleman and MeierAugenstein, 2014), interspersed among each of two batch analysis. Measurement accuracy was checked by the analysis of two triplicates of the secondary reference material for each batch, from which analytical variance was calculated (standard deviation of 0.21‰ for the first batch and 0.08‰ for the second). Machine drift was compensated in each batch (LIE-SIIAF, 2018).

2.6. In-situ soil CO_2 flux measurements

At the beginning of the exposure period, soil CO_2 flux measurements were performed *in-situ*, underneath each lichen transplant (Furnas and reference sites), in order to assess localized soil degassing intensity, since lichen transplants were not placed exactly in the same coordinates as the points surveyed previously by Viveiros et al., 2010 for the E-type estimate map of soil CO_2 flux on Furnas caldera (Fig. 2). All measurements were done in a single summer day, in stable weather conditions, to minimize the influence of varying meteorological parameters on the soil gas flux (Viveiros et al., 2008; Viveiros et al., 2009), although it is still conditioned by soil permeability determined by soil properties. The portable CO_2 flux station (Ref. WS1214), similar to the one used by Viveiros et al., 2010, and manufactured by WEST Systems, is based on the accumulation chamber method (Norman et al., 1992; Chiodini et al., 1998), and is equipped with a LICOR LI-800 infrared CO_2 detector (L-IR) that has a full scale set at 20000 ppm. Soil temperature at 25 cm depth was determined with a Testo 925 thermometer plus type K temperature probe.

2.7. $\delta^{34}S$ interpolations

All retrieved lichen transplants (n = 39) were considered statistically independent samples. Its $\delta^{34}S$ values, measured on lichen transplants (Fig. 3), were spatially modeled using geostatistics tools (Fig. 4). After calculating the empirical semi-variogram using a 150 m step, a Gaussian function with zero nugget effect and a major range of 900 m in the SW-NE (69°) direction was found to fit well the data. The nugget effect was determined using the information from the reference site, where 3 samples were kept simultaneously in the same location. The semi-variance between these 3 samples was very low, suggesting that the error of measure was negligible, thus supporting the use of a nugget of zero. This further supported the dismissal of sample replication per site, as differences of $\delta^{34}S$ values in lichen thalli collected in the same site are small and associated to lichen age (Yun et al., 2010) (which was controlled for by homogenizing the lichen population prior to transplant) and little or no fractionation occurs to $\delta^{34}S$ after it has been assimilated by lichens and, therefore, lichens retain the S isotopic signature of the surrounding atmosphere (Barros et al., 2015). Anisotropy was incorporated into the model with a minor range of 415 m in the SE-NW direction (159°). This function was then used to interpolate $\delta^{34}S$ within the study area using ordinary kriging.

2.8. Risk analysis

To determine and spatially model the habitational risk areas of chronic exposure to outdoors airborne hydrothermal emissions, the entire polygonal habitational area of Furnas village, overlapping with the $\delta^{34}S$ interpolation map area, was delimited (according to Google Maps aerial view) and then divided in a 30 × 30 m squared grid. The relative area, within each 900 m^2 cell, occupied by habitations (from 0% = no habitations up to 100% = entirely occupied by habitations) was calculated as a proxy of population concentration. All cells with non-zero values for the relative habitational area were intersected with the vectorized $\delta^{34}S$ interpolation map to calculate their mean $\delta^{34}S$ values, used as the tracer of airborne hydrothermal emissions. The final determination of the chronic exposure risk levels was done relating both data per cell (Fig. 5A). More specifically, risk was then categorized in six levels, from reduced to very high, defined by the pairing of exposure and hazard risk components (Rodricks, 1992) as follows: the percentage of the area occupied by habitations within each cell (exposure component) in three intervals of increasing habitational occupation (<40%;

40%–70%; 70%–100%); the mean $\delta^{34}S$ value predicted for each cell (hazard component), categorized in two intervals of $\delta^{34}S$ values (>9.5‰; <9.5‰), above and below the overall mean $\delta^{34}S$ of Furnas lichen transplants. This value was assigned as a sensible halfway cut point defining a threshold of manifest hydrothermal influence, since igneous rocks of primary origin can present S isotopic compositions ranging between −10‰ to +10‰ (Thode, 1991) and past measurements of $\delta^{34}S$ on hydrothermal fumaroles, not only at Furnas fumarolic fields (Wilson, 1966; Ferreira and Oskarsson, 1999), were <10‰ and lean towards values closer to zero. The highest risk level was attained in cells combining the highest % of the habitational area and the lowest $\delta^{34}S$ values (Fig. 5A). It was assumed that cells without habitational area have a null risk of human chronic exposure since the prolonged exposure component equals zero. After pairing the risk components, cells were aggregated into polygons by risk level and intersected with the initial land-cover polygon to derive a more realistic mapping of the habitational risk areas (Fig. 5B).

Statistical analysis was carried out with Microsoft Office Excel (Microsoft Corp., 2018) and IBM SPSS Statistics v. 25 (IBM Corp, 2017), while geospatial modelling and mapping were accomplished using ESRI ArcMap™ v. 10.6 (ESRI, 2018) and OSGEO QGIS v3.4.1 (Open-Source Geospatial Foundation Project, 2018).

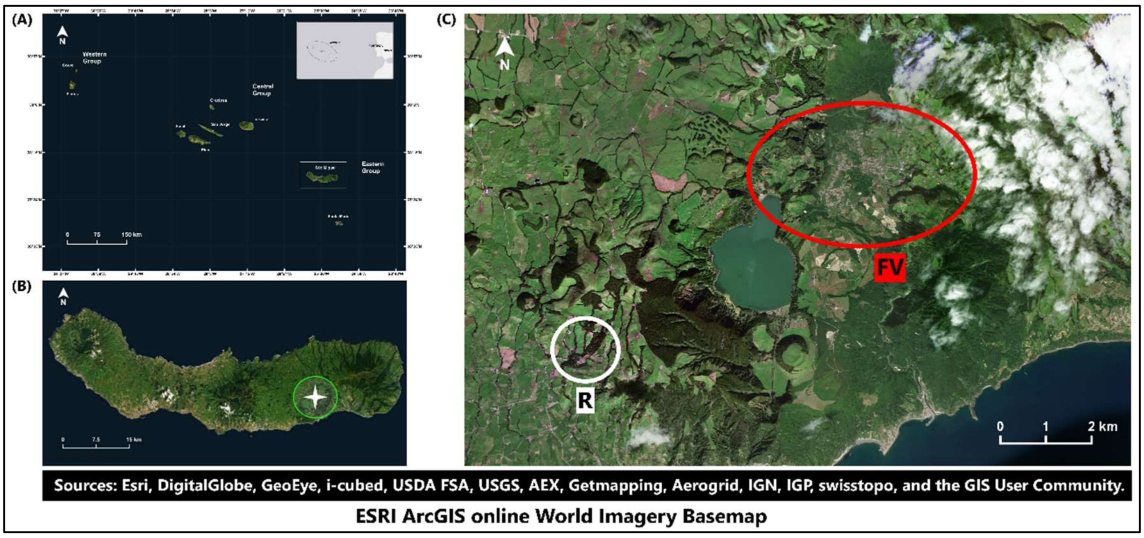
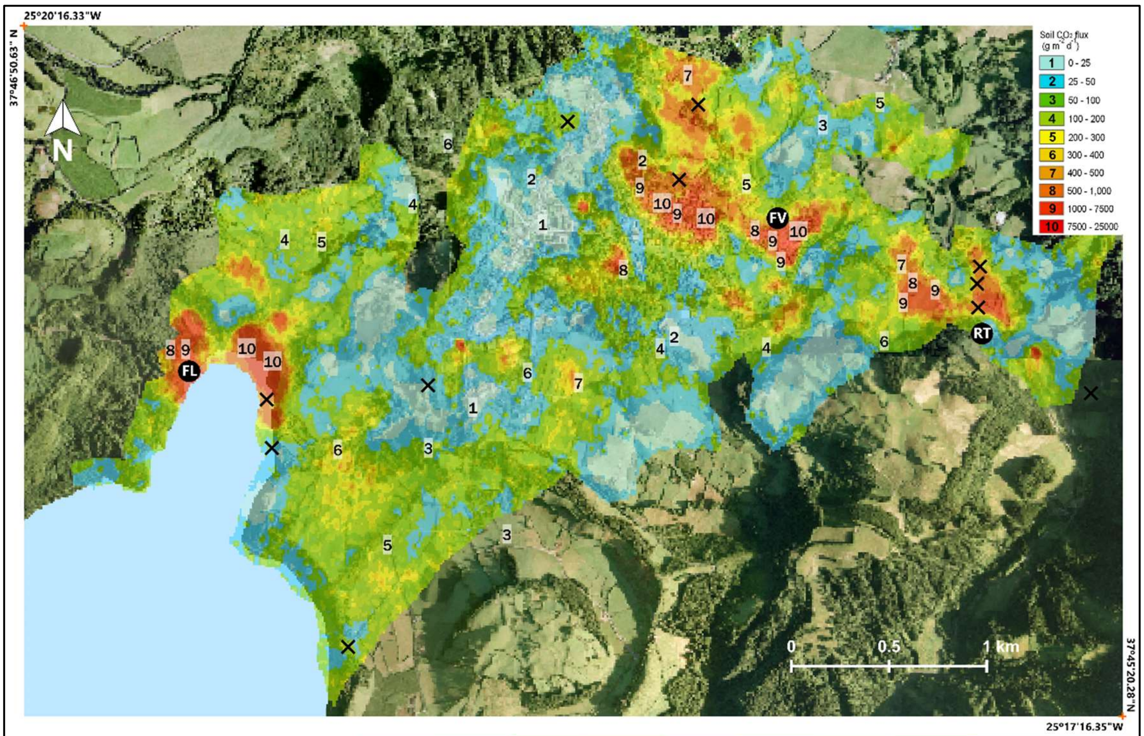


Figure 1. (A) Location of São Miguel island in the Azores archipelago. Geocoordinates CRS datum: WGS 84. (B) Location of Furnas volcano in São Miguel island (star). (C) Location of the study site (FV – Furnas village) and reference site (R – Recreation forest reserve of Cerrado dos Bezerros, Ponta Garça village) in São Miguel island. Land cover surrounding Furnas village comprises pastures, cultivation fields and forests. Basemap aerial view backgrounds by ESRI ArcGIS online. "World Imagery" [basemap]. "World Imagery Map". Last updated December 14, 2018. https://www.arcgis.com/home/item.html?id=10df2279f9684e4a9f6a7f08febac2a9. Attribution information to both ESRI and other data providers shown in the figure.



CO_2 Flux class number	Reference	1	2	3	4	5	6	7	8	9	10
Expected CO_2 Flux class range ($g/m^2.d$)	NA	0-25	25-50	50-100	100-200	200-300	300-400	400-500	500-1000	1000-7500	7500-25000
Observed average soil CO_2 Flux values ($g/m^2.d$)	7.47	31.31	28.78	39.30	34.30	31.00	31.71	48.99	650.67	221.49	1189.74
Number of transplants recovered after 6 months	3	2	3	3	4	4	4	3	4	7	5

Figure 2. Spatial distribution of the lichen transplants placed at the study area on Furnas volcano caldera, divided between 10 classes of soil CO_2 flux, estimated accordingly to the soil CO_2 flux degassing map for Furnas caldera by Viveiros et al. (2010). This is an interpolated map where the expected values are estimated at any considered location. Transplants in locations within areas classified within the same soil CO_2 flux class are identified with the same number. Lost transplants (from the initial 50) are marked with an X. Average *in-situ* soil CO_2 flux values of reference samples and Furnas transplants belonging within each of the 10 classes are shown in the appended table. (FL) Furnas lake fumarolic field. (FV) Furnas village fumarolic field. (RT) Ribeira dos Tambores fumarolic field. Geocoordinates CRS datum: WGS 84/Pseudo-Mercator.

3. Results and discussion

3.1. In-situ soil CO_2 flux

In-situ soil CO_2 flux values were measured at the ground level for the same sampling sites used for lichen transplants. As expected, *in-situ* soil CO_2 fluxes were higher on the sites of the study area than on the reference site. Furthermore, the hydrothermal degassing gradient occurring on the study area was revealed, as *in-situ* soil CO_2 fluxes were positively correlated with the expected class ranges ($n = 39$; $\rho = 0.419$, $p = 0.008$) and negatively correlated with the distance to the closest fumarolic field ($n = 39$; $\rho = -0.426$, $p = 0.007$). However, the biogenic CO_2 flux threshold of 50 $g/m^2.d$ (above which the CO_2 flux is considered to be fed also by the hydrothermal source) was only surpassed in locations where a tenfold soil CO_2 flux was expected (500 g/m^2.d) (Fig. 2). Besides inter-variability fluctuation, lower than expected soil CO_2 flux values are most likely explained by heterogeneities in soil properties, as highlighted by Viveiros et al. (2010) or even by slight changes on the degassing pattern explained by the more than 8 years difference between the two surveys. Those authors observed a higher nugget effect on soil CO_2 flux surveys made near the main fumarolic fields, where soil alteration causes large differences in the permeability at very small scale, when compared with general surveys throughout the volcano caldera. This implies that measurements performed on adjacent points can display considerable disparity on account of the soil permeability and thus misrepresent the real soil CO_2 degassing magnitude of the surrounding area, especially on the fumarolic fields which are, coincidentally, high-volume sources of hydrothermal emissions with a tendency to low soil permeability. Taking this into consideration, lichen biomonitoring is a fundamental complement to *in-situ* soil CO_2 flux surveys to assess the outdoors chronic exposure to airborne hydrothermal emissions and build maps of the risk areas with high spatial resolution, as results further reveal.

3.2. S isotopic ratio

$\delta^{34}S$ is positively and strongly correlated with the distance to the closest fumarolic field (n = 39; $\rho = 0.855$, $p < 0.001$). A regression model (using a logarithmic function with least squares estimator) was found to best suit the data concerning the relationship between $\delta^{34}S$ values on Furnas lichen transplants and distance to the closest fumarolic field (Fig. 3). $\delta^{34}S$ is also negatively correlated with *in-situ* soil CO_2 flux (n = 39; $\rho = -0.589$, $p < 0.001$).

$\delta^{34}S$ values tend to decrease towards 0‰ on Furnas transplants with proximity to the closest fumarolic field. $\delta^{34}S$ values slightly below 0‰ have already been reported by Ferreira and Oskarsson (1999) for the S isotopic composition of fumarolic discharges from Furnas lake (−0.7‰) and Furnas village (−1.8‰) fumaroles. Furthermore, Cruz et al. (1999) also attributed decreased $\delta^{34}S$ values of Furnas carbonated and thermal waters (closer to 0‰) to the leaching of sulphate from volcanic rocks, contrasting with the S isotopic ratios of cold spring waters with low dissolved solids, which tend towards seawater levels (around 25‰). Similarly, the lower $\delta^{34}S$ values in lichen transplants near the fumarolic fields contrast with the higher $\delta^{34}S$ values in samples from the reference site and lichen transplants further from the fumarolic fields, indicating that the prevailing atmospheric S isotopic signature is higher, characterized by $\delta^{34}S$ values around 13–14‰. Bearing in mind that Furnas volcano caldera and the reference site are at 3.5 km and 2.6 km of the ocean, respectively, these background values are consistent with the prevailing oceanic influence, detectable at 3 to 5 km and up to 10 km from the shoreline (Barros et al., 2015), by biogenic dimethylsulfide (DMS) marine aerosols ($\delta^{34}S$ of 15.6‰ ± 3.1‰) (Calhoun et al., 1991). As S terrestrial sources are usually more depleted in the rarer ^{34}S isotope than marine sources (McArdle and Liss, 1995; Wadleigh and Blake, 1999; Barros et al., 2015), and little to no fractionation occurs to $\delta^{34}S$ after assimilation by lichens (Batts et al., 2004; Bačkor and Loppi, 2009; Barros et al., 2015), lichen uptake of volcanogenic sulfur, continuously released along with the airborne hydrothermal steams, was expressed in $\delta^{34}S$ values decreasingly below the prevailing background, marine-like $\delta^{34}S$ values measured in reference samples and transplants furthest from Furnas fumarolic fields. Naturally, a greater availability of volcanogenic S for lichen uptake is expected in the air near the fumarolic fields, but as the fumarolic emissions tendentially dissipate over distance, the volcanogenic S is increasingly diluted with the prevailing atmospheric S. This tendency is in accordance with Barros et al. (2015), who found lower $\delta^{34}S$ values in lichens around anthropogenic sources in a coastal industrial area, contrasting with the prevailing background marine influence. The same contrasting effect of the fumarolic sources is visually noticeable by spatial modelling by interpolations of $\delta^{34}S$ values of lichen transplants within the study area (Fig. 4).

In short, like in other studies tracing atmospheric S sources with lichens (Barros et al., 2015; Wadleigh and Blake, 1999), these results, from a volcanic site, confirm that isotopic $\delta^{34}S$ is a suitable tracer to reveal the time-integrated dispersal of airborne hydrothermal emissions around fumarolic fields on a volcanic environment.

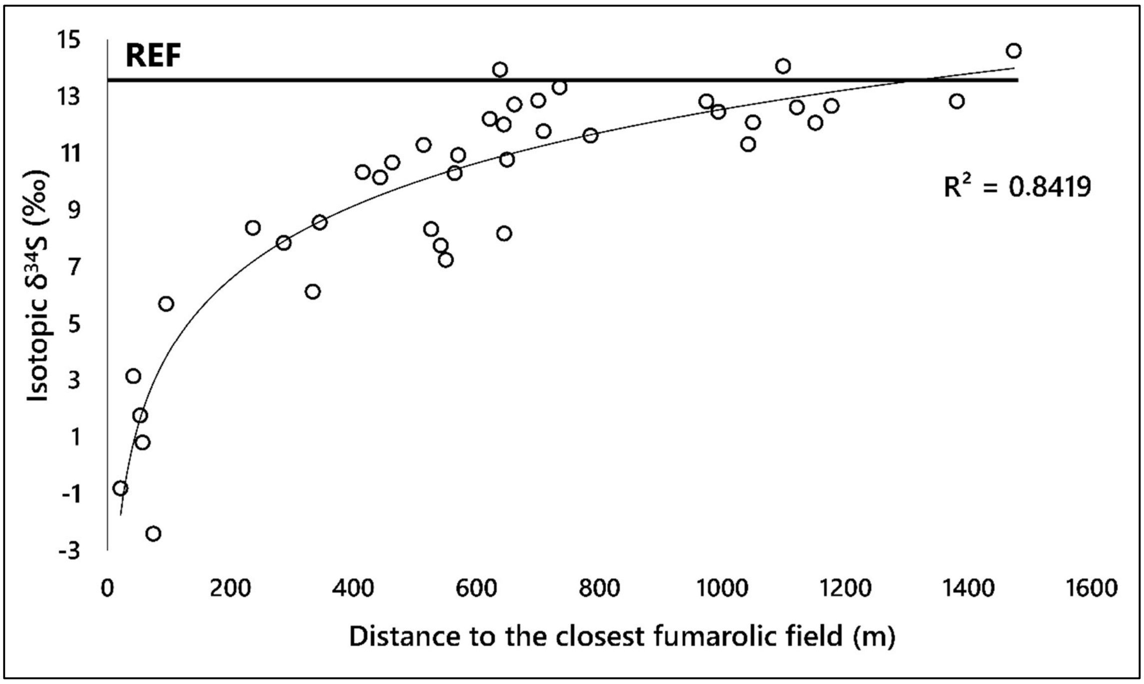


Figure 3: Logarithmically best fitted scatter plot of $\delta^{34}S$ values, measured on Furnas lichen transplants, in function of the linear distance to the closest fumarolic field. Background average of the reference samples is shown by the REF line. Regression equation for $\delta^{34}S$: $y = 3.7157 \ln(x) - 13.119$; $R^2 = 0.8419$.

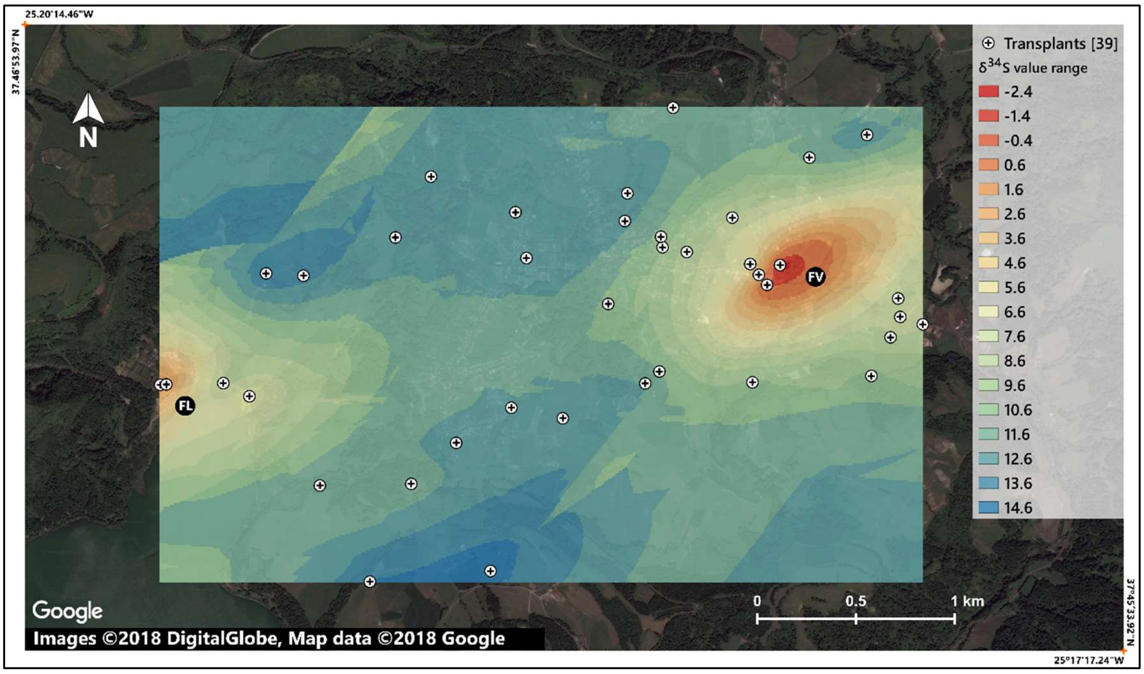


Figure 4: Spatial modelling of S isotopic signatures by interpolation of $\delta^{34}S$ isotopic values measured on Furnas lichen transplants, obtained by ordinary kriging interpolations. Lichen transplants are marked with crossed white dots. FL) Furnas lake fumarolic field; FV) Furnas village fumarolic field. Basemap aerial view background belongs to Google Maps, which includes the attribution information to both Google and third-party data providers, accordingly to Google Maps attribution guidelines (Google LLC, 2018). Geocoordinates CRS datum: WGS 84/Pseudo-Mercator.

3.3. Chronic exposure risk assessment

By combining $\delta^{34}S$ data measured in lichen transplants and Furnas village habitational areas for chronic exposure risk level determination (Fig. 5A), the habitational areas could be spatially modeled by their increased risk level (Fig. 5B). Almost 26% of Furnas village habitational areas, within the space of the study area under lichen transplant coverage, mostly around the Furnas village fumarolic field, are at high or very high risk of outdoors chronic exposure to volcanogenic air pollution, as traced by $\delta^{34}S$ signature. This methodology is employable in other inhabited volcanic areas of the world, even when detailed mapping of the degassing structures (through extensive soil geochemistry surveys) are unavailable, as lichens are able to reveal the time-integrated and spatial patterns of atmospheric dispersal of airborne volcanogenic emissions, although the spatial resolution and risk level precision can be enhanced depending on the density of lichen transplant sampling and detail of the mapping of habitational areas. Nevertheless, there are several soil degassing areas without sulfur emissions (Viveiros et al., 2010), thus the development of even more comprehensive and realistic models, assessing human chronic exposure risk to volcanogenic emissions, should conjugate both outdoor (lichen biomonitoring) and indoor (CO_2 and ^{222}Rn surveys) susceptibility maps. Exposure routes for the different hazards should be considered, as villagers circulate between indoor and outdoor spaces, singling out areas where human health studies should be focused and corresponding protective or mitigating measures for each exposure route should be applied to ensure public health safety.

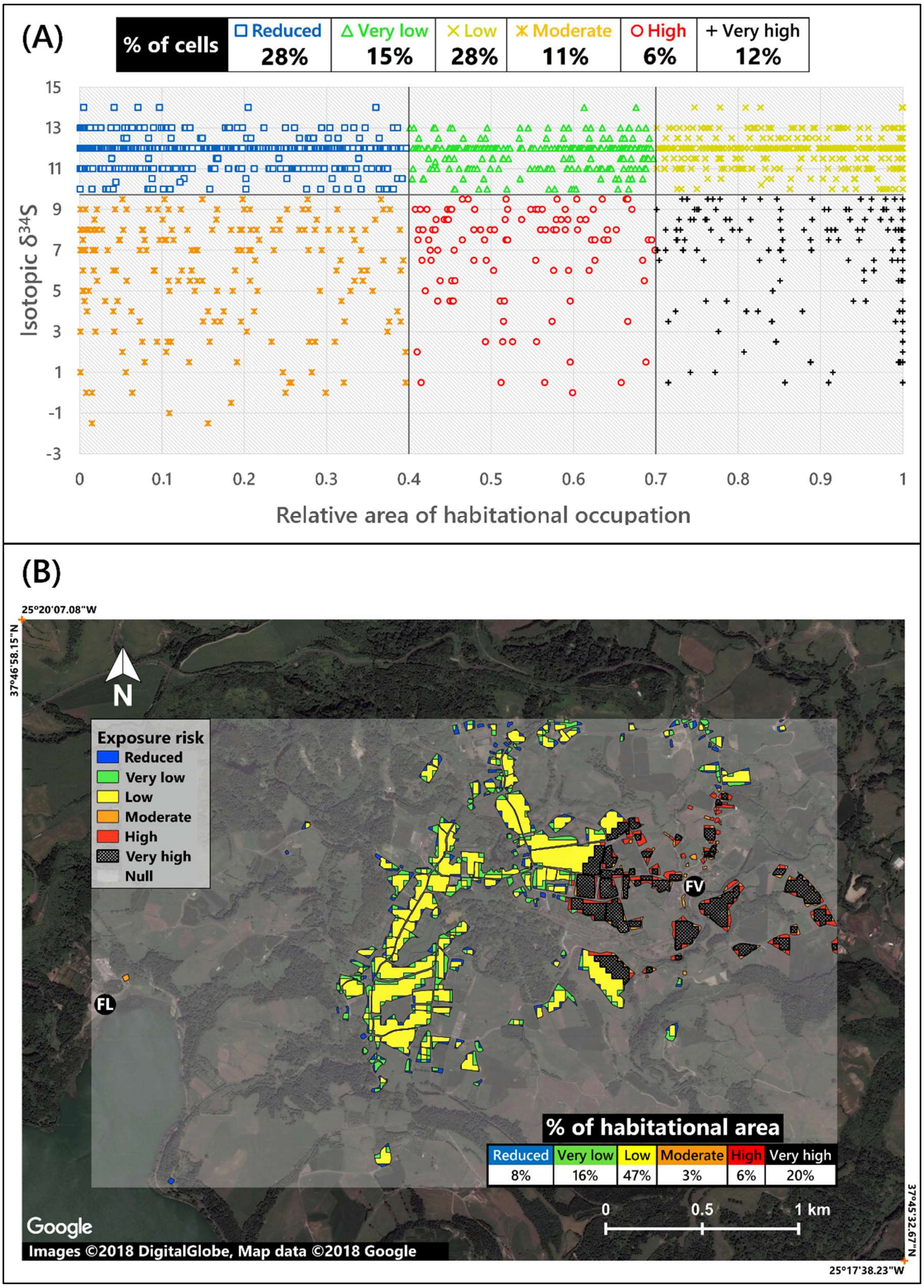


Figure 5: (A) Scatter plot of Furnas village habitational area within the space of the study area under lichen transplant coverage. Each symbol is a 900 m^2 square cell representing an area unit plotted accordingly to paired values provided by their mean interpolated isotopic $\delta^{34}S$ values and the relative area (greater than zero and up to one) occupied by habitations. The percentages of cells classified within each risk level of chronic exposure are shown in the top table. The risk level on cells without habitational area was assumed as null, therefore they are not represented. (B) The resulting spatial modelling, with the polygonal representation and risk level classification of Furnas village habitational areas, within the space of the study area under lichen transplant coverage, on a 900 m^2 squared cell resolution, according to their risk level of chronic exposure. The percentage of the habitational areas included within each risk level is shown in the bottom table. Remaining areas with no habitations are considered to have a null risk. FL) Furnas lake fumarolic field; FV) Furnas village fumarolic field. Basemap aerial view background belongs to Google Maps, which includes the attribution information to both Google and third-party data providers, accordingly to Google Maps attribution guidelines (Google LLC, 2018). Geocoordinates CRS datum: WGS 84/Pseudo-Mercator.

4. Conclusion

The ability of lichens to retain the specific volcanogenic S isotopic signature, following a prolonged exposure to hydrothermal emissions in a volcanically active area, like Furnas village, in a pattern consistent with the soil degassing gradient and distance to fumarolic fields, demonstrates their suitability as ecological indicators for spatially tracing and temporally integrating the atmospheric dispersal of airborne volcanogenic emissions in outdoor conditions, under the influence of uncontrolled atmospheric fluctuations. Lichen biomonitoring data is a valuable and cost-saving complement to conventional monitoring data for realistically assessing the risk level of chronic exposure of human populations living in volcanic areas of the world, allowing to spatially model the risk areas with high resolution. Correlation with population health outcomes, like chronic respiratory conditions, or human biomonitoring data, might shed light regarding the critical risk level of chronic exposure translating to adverse health effects.

Credit authorship contribution statement

<ins>Filipe Bernardo</ins>: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. <ins>Pedro Pinho</ins>: Methodology, Formal analysis, Writing - review & editing, Visualization. <ins>Paula Matos</ins>: Methodology, Writing - review & editing. <ins>Fátima Viveiros</ins>: Methodology, Investigation, Resources, Writing - review & editing. <ins>Cristina Branquinho</ins>: Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. <ins>Armindo Rodrigues</ins>: Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition. <ins>Patricia Garcia</ins>: Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Declaration of competing interest

The authors declare no competing interests.

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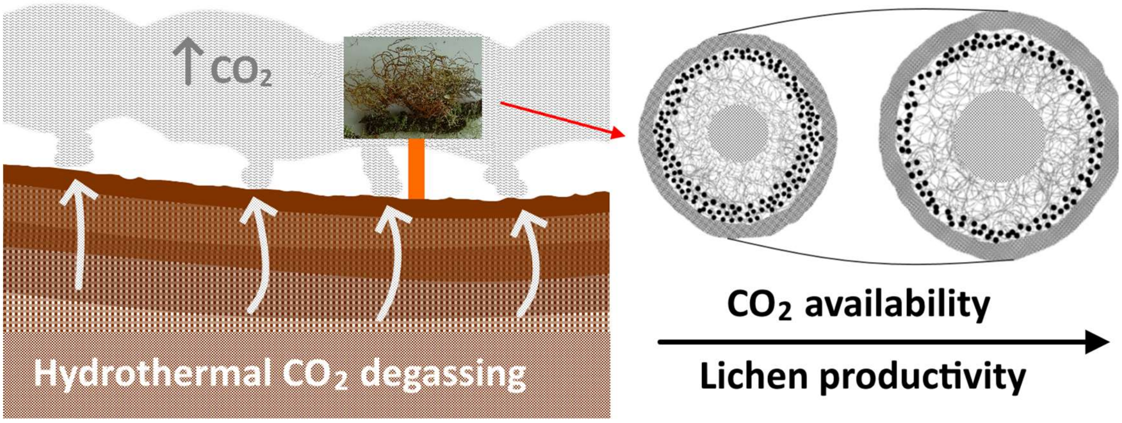
3.2. Thallus structural alterations in green-algal lichens as indicators of elevated CO_2 in a degassing volcanic area



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Thallus structural alterations in green-algal lichens as indicators of elevated CO_2 in a degassing volcanic area

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ABSTRACT

We hypothesize that the thalli of the green-algal, CO_2-sensitive lichen *Usnea rubicunda* (Stirton) undergo significant structural alterations as a biological response indicating increased CO_2 emissions. To test that, *U. rubicunda* thalli were transplanted from a reference site with no volcanism into 36 sites distributed within the volcanically active Furnas caldera, encompassing the gradient of soil diffuse CO_2 degassing areas (700 t d^{-1} of hydrothermal CO_2). After 6 months of exposure in similar macroclimatic conditions, both Furnas transplants and samples kept at the reference site were retrieved for histology to assess thalli structure and the proportion between the symbionts. On average, cross-sections of Furnas thalli were significantly thicker than reference, owing mostly to the fungal layers of medulla and central cord. The latter occupied a significantly greater than reference relative volumetric density despite a smaller than reference relative percentage of algal occupation on Furnas thalli. These results reveal a positive and time-integrated response of *U. rubicunda* to the greater availability of CO_2 from hydrothermal emissions in the volcanic environment, translated in greater efficiency of the algae in sustaining the fungal biomass. Histomorphometric structural alterations in the heteromerous thalli of *U. rubicunda* are suitable response biomarkers with potential to indicate a global rise of CO_2 levels in natural environments.

1. Introduction

Lichens are outstanding ecological indicators of the effects of global change in ecosystems (Branquinho et al., 2015), due to their poikilohydric and poikilothermic nature and limited control on water and gas exchange (Batts, Calder, & Batts, 2004; Garty, 2001). Lichens are a symbiosis between a producer, the photosynthetic partner (algae and/or cyanobacteria) and a consumer, the fungal partner, integrated into the same organism. Lichens have a differentiating interaction with the air, depending almost exclusively on atmospheric input for water and mineral nutrition and passively obtaining CO_2 without stomatal regulation (Batts et al., 2004), thus more directly reflecting changes in atmospheric composition (Branquinho et al., 2015). Lichens are substantial carbon sinks in the environment (Seminara, Fritz, Brenner, & Pringle, 2018). Approximately 40 to 50% of lichens dry mass consists of carbon (Green, Nash, & Lange, 2008), mostly retained on the fungal cell wall polysaccharides and extracellular secondary carbon metabolites (Ding, Zhou, & Wei, 2013; Palmqvist, Dahlman, Jonsson, & Nash, 2008). Recent estimates of global carbon net uptake from the atmosphere by cryptogamic covers, mostly comprised of lichens and bryophytes, amount to up to 6%–7% (3.3–3.9 Gt yr^{-1}) of the terrestrial net primary productivity (Elbert et al., 2012; Porada, Weber, Elbert, Pöschl, & Kleidon, 2013). Thus, lichens can play a notable role in the global biogeochemical cycles of carbon, most noticeably in drier desert habitats where they are key species (Elbert et al., 2012). Hence, it remains of great interest to assess if the effects of the globally rising atmospheric CO_2 concentrations from anthropogenic emissions significantly increase the primary productivity of lichen communities on terrestrial ecosystems (Nash, 1996), paralleling the plant CO_2 fertilization which has been attributed primarily to the stimulating effect of rising atmospheric CO_2 on photosynthesis in vascular plants (Kondo et al., 2018). Considering the peculiar dual nature of lichens, it is also interesting to understand how such changes in productivity are reflected in the relationship between the producer and the consumer symbionts.

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Abstract

We hypothesize that the thalli of the green-algal, CO_2-sensitive lichen *Usnea rubicunda* (Stirton) undergo significant structural alterations as a biological response indicating increased CO_2 emissions. To test that, *U. rubicunda* thalli were transplanted from a reference site with no volcanism into 36 sites distributed within the volcanically active Furnas caldera, encompassing the gradient of soil diffuse CO_2 degassing areas (700 $t.d^{-1}$ of hydrothermal CO_2). After 6 months of exposure in similar macroclimatic conditions, both Furnas transplants and samples kept at the reference site were retrieved for histology to assess thalli structure and the proportion between the symbionts. On average, cross-sections of Furnas thalli were significantly thicker than reference, owing mostly to the fungal layers of medulla and central cord. The latter occupied a significantly greater than reference relative volumetric density despite a smaller than reference relative percentage of algal occupation on Furnas thalli. These results reveal a positive and time-integrated response of *U. rubicunda* to the greater availability of CO_2 from hydrothermal emissions in the volcanic environment, translated in greater efficiency of the algae in sustaining the fungal biomass. Histomorphometric structural alterations in the heteromerous thalli of *U. rubicunda* are suitable response biomarkers with potential to indicate a global rise of CO_2 levels in natural environments.

Highlights:

- *U. rubicunda* transplanted to CO_2 degassing volcano displays structural alterations.
- Their heteromerous thalli is significantly thicker on the exposed samples.
- Fungal central cord is proportionally increased despite relatively less algae.
- Histomorphometric measures are time-integrated response biomarkers.
- Lichens can be suitable bioindicators of increased CO_2 in natural environments.

Keywords: *Usnea rubicunda*; Volcanism; Hydrothermal emissions; Carbon dioxide; Histology; Biomarkers.

1. Introduction

Lichens are outstanding ecological indicators of the effects of global change in ecosystems (Branquinho et al., 2015), due to their poikilohydric and poikilothermic nature and limited control on water and gas exchange (Batts, Calder, & Batts, 2004; Garty, 2001). Lichens are a symbiosis between a producer, the photosynthetic partner (algae and/ or cyanobacteria) and a consumer, the fungal partner, integrated into the same organism. Lichens have a differentiating interaction with the air, depending almost exclusively on atmospheric input for water and mineral nutrition and passively obtaining CO_2 without stomatal regulation (Batts et al., 2004), thus more directly reflecting changes in atmospheric composition (Branquinho et al., 2015). Lichens are substantial carbon sinks in the environment (Seminara, Fritz, Brenner, & Pringle, 2018). Approximately 40 to 50% of lichens dry mass consists of carbon (Green, Nash, & Lange, 2008), mostly retained on the fungal cell wall polysaccharides and extracellular secondary carbon metabolites (Ding, Zhou, & Wei, 2013; Palmqvist, Dahlman, Jonsson, & Nash, 2008). Recent estimates of global carbon net uptake from the atmosphere by cryptogamic covers, mostly comprised of lichens and bryophytes, amount to up to 6%–7% (3.3–3.9 Gt yr^{-1}) of the terrestrial net primary productivity (Elbert et al., 2012; Porada, Weber, Elbert, Pöschl, & Kleidon, 2013). Thus, lichens can play a notable role in the global biogeochemical cycles of carbon, most noticeably in drier desert habitats where they are key species (Elbert et al., 2012).

Hence, it remains of great interest to assess if the effects of the globally rising atmospheric CO_2 concentrations from anthropogenic emissions significantly increase the primary productivity of lichen communities on terrestrial ecosystems (Nash, 1996), paralleling the plant CO_2 fertilization which has been attributed primarily to the stimulating effect of rising atmospheric CO_2 on photosynthesis in vascular plants (Kondo et al., 2018). Considering the peculiar dual nature of lichens, it is also interesting to understand how such changes in productivity are reflected in the relationship between the producer and the consumer symbionts. Connecting these aspects with CO_2 availability would allow lichens to serve as integrated indicators of the effects of atmospheric CO_2.

However, field studies addressing the response of lichens to prolonged exposure to elevated CO_2 in natural conditions are few and have not clearly demonstrated a greater benefit to lichen productivity, at least for naturally occurring lichens sampled *in-situ* (e.g. Balaguer et al., (1999). In order to further address this gap, this study alternatively resorts to lichen transplantation, during a delimited exposure period, to determine whether a lichen species containing *Trebouxia* green algae, known to be sensitive to changes in external concentrations of CO_2 (Balaguer et al., 1999; Cowan, Lange, & Green, 1992), displays similar thalli structural response once exposed from normal to elevated CO_2 levels. The organization and structure of the symbiotic lichen thalli are greatly modifiable by environmental conditions, leading to eco-morphological differentiation associated with habitat adaptation (Osyczka, Boroń, Lenart-Boroń, & Rola, 2018). Thus, histomorphometric approaches can be proposed to test the hypothesis that significant structural alterations occur in green-algal lichen thalli as primary responses to elevated CO_2 in the atmosphere. Since acclimation is commonly observed in photosynthetic organisms when exposed to elevated CO_2 (Ainsworth & Long, 2005), we propose to track the structural responses of lichens following the transplantation from a site with background CO_2 atmospheric concentrations to a site with elevated CO_2, both sharing a temperate climate of non-limiting conditions of mild temperatures and high air humidity.

Specifically, the fruticose green-algal lichen species *Usnea rubicunda* (Stirton) was transplanted from a pristine reference site, with no volcanic activity, to multiple spots within Furnas volcano caldera, in São Miguel island (Azores), an actively degassing volcanic area characterized by abundant CO_2 emissions of hydrothermal origin, which served as the natural source of elevated CO_2. Active volcanoes are important natural sources of CO_2 by releasing high volumes to the atmosphere during eruptive episodes as well as quiescent periods (Chiodini, Cioni, Guidi, Raco, & Marini, 1998), forming peculiar habitats strongly influenced by degassing phenomena. Therefore, volcanic areas are privileged natural laboratories to investigate how lichens deal with the abundance of CO_2. After six months of field exposure, the relative volumetric density and thicknesses of the thalline layers, as well as the relative abundance of algal cells, were measured in cross-sections of marginal portions of the thalli of exposed transplants as well as reference samples kept on the reference site, in order to determine the extent of histological thallus modification. These structural alterations could be suitable response biomarkers in *U. rubicunda* concerning the effects of increased atmospheric CO_2 levels under nonlimiting climatic conditions in natural environments, revealing specifically the outcome of CO_2 enrichment on integrated lichen productivity and on the interaction between the symbionts.

2. Materials and methods

2.1. Study area and reference site

Figure 1 displays the locations of Furnas volcano and the reference site. Soil diffuse degassing emissions account for over 90% of the total hydrothermal CO_2 output from Furnas volcano, with an estimate of at least 734 t d^{-1} of CO_2 within Furnas caldera (Ferreira et al., 2005; Pedone et al., 2015; Viveiros et al., 2010). Land cover in Furnas village comprises mostly pastures, cultivation fields and forests, whereas traffic, urban and industrial activities are very low. Therefore, since CO_2 is the main gas released by soil diffuse degassing and fumaroles (Viveiros et al., 2009), hydrothermalism is the main source of airborne CO_2 at Furnas.

The reference site is a pristine non-active volcanic area, corresponding to a forest reserve located only 3 km apart from Furnas, outside the volcano caldera (Figure 1), sharing similar macroclimatic conditions during the year and likewise low traffic and no industrial activity. Being located in an island, at < 5 km from the ocean, both Furnas and reference site enjoy a temperate climate, with mild temperatures rarely below 10 °C and above 30 °C, seasonal rainfall patterns reaching a mean annual precipitation of almost 2000 mm (Marques, Zêzere, Trigo, Gaspar, & Trigo, 2008) and constantly high air humidity around 80%, including frequent fog and dew. Lichens are ensured regular moistening in either location to be photosynthetically active, which is one major limiting factor to lichen productivity as poikylohydric organisms (Lange & Green, 1996; Nash, 1996).

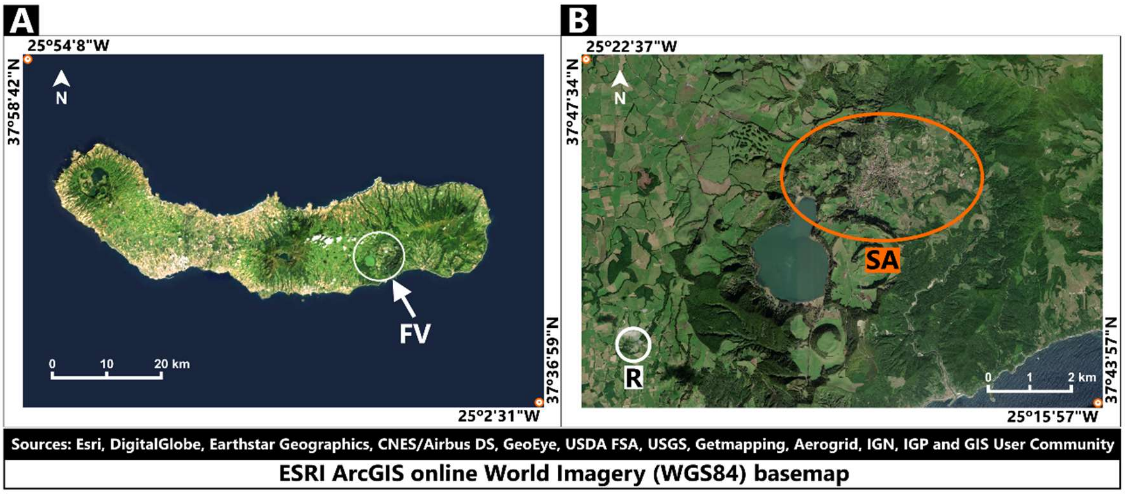

Figure 1. (A) Location of Furnas volcano (FV) in São Miguel island. (B) Location of the study area (SA - within Furnas volcano caldera) and reference site (R – Recreation forest reserve of Cerrado dos Bezerros, Ponta Garça village). Geocoordinates CRS datum: WGS 84. Basemap aerial view backgrounds by ESRI ArcGIS online. "World Imagery (WGS84)". Last updated 06 January 2020. https://www.arcgis.com/home/item.html?id=898f58f2ee824b3c97bae0698563a4b3. Attribution information to both ESRI and other data providers shown below the figure.

2.2. Sampling design

Thirty-six suitable sampling sites within Furnas volcano caldera were chosen for the placement of lichen transplants in outdoor conditions. Their locations were chosen ensuring a minimum distance of 20 m to the nearest transplant, to cover homogeneously the study area while also encompassing areas from low to high soil CO_2 diffuse degassing intensity (following the soil CO_2 flux class ranges defined and mapped by Viveiros et al., 2010 for Furnas caldera). The average distance between spots was 186 m. One lichen transplant bag was placed per spot. The exposure period of all lichen transplants lasted for 6 months, from late July 2017 to January 2018, progressing from the end of the dry season into the rainy season.

During the same period, 8 additional lichen transplant bags, receiving identical experimental handling as Furnas transplants, were kept on 8 different tree trunks at the reference site, assuredly not exposed to hydrothermal emissions, providing reference values for the measurements on the lichen thalli.

2.3. Lichen transplants

Lichen transplantation involves the relocation of specimens naturally occurring in a relatively unimpacted environment to the area of interest, subject to air pollution, for a fixed exposure period (Mikhailova, 2002; Williamson, Purvis, Mikhailova, Spiro, & Udachin, 2008). This strategy has practical advantages over *in-situ* sampling of pre-existing lichens, such as repeatability, greater flexibility in spatial distribution, well-defined exposure time and ensuring exposure free condition at the beginning of the experiment (Caggiano, Trippetta, & Sabia, 2015).

Each lichen transplant bag placed at Furnas caldera lodged several *U. rubicunda* thalli inside a porous stretchable nylon net to allow free airflow. These lichens used for transplantation to the study area were natively occurring on *Cryptomeria japonica* trunks of the reference site. There is no record of volcanic emissions at the reference site. Within 8 days after collection from the reference site, the lichen transplant bags were moved to the corresponding spots of the study area, attached on available supports such as tree trunks and posts, promoting as much as possible the unconstrained and unconfined ambient exposure in field conditions. Considering CO_2 is denser than the air, tending to accumulate in poorly ventilated or low-lying zones (Viveiros et al., 2009), transplant bags were placed at 1.5 m of height on the support, preventing an unrealistic exposure to airborne CO_2 potentially accumulated at ground level, as epiphytic lichens can occur at greater heights, allowing some CO_2 dilution into the circulating air. Furthermore, each transplant bag was placed in the open, as clear as possible from surrounding obstacles like dense tree foliage, that could block air circulation, sunlight and rainfall, endeavoring to minimize as much as possible microclimatic differences at each spot.

U. rubicunda was selected as the lichen species for transplantation because, besides being easily identifiable, easy to collect, handle and transplant, this lichen species occurs natively in the vicinity of Furnas fumarolic fields, ensuring simultaneously a degree of tolerance to hydrothermal air pollution. In addition, due to its shrubby fruticose growth-form (Fig. 2A), translating to a large surface area per unit of dry weight, greater interaction with the surrounding air and enhanced particle interception is expected (Branquinho et al., 2008; Garty, 2001).

2.4. Lichen sampling processing

The 36 Furnas transplants and 8 reference samples were simultaneously retrieved and processed within a few days. Previously to laboratory processing, damaged portions were excluded. All 44 lichen bags contained enough healthy material to proceed forward so that only visibly unscathed and healthy-looking apical lichen portions were considered. In more detail, multiple marginal tips of 1 cm in length were arbitrarily collected from the healthy-looking outermost branches of several *U. rubicunda* thalli of each transplant bag. This is because the edges of the lichen thalli are the youngest and coincidentally the most physiologically and metabolically active, especially in larger lichens (Bargagli & Barghigiani, 1991; Godinho, Verburg, Freitas, & Wolterbeek, 2009; Loppi, Nelli, Ancora, & Bargagli, 1997; Nimis, Andreussi, & Pittao, 2001; Seminara et al., 2018), corresponding to the thallus elongation zones. Thus, the selected younger lichen portions had the greatest possible lifetime overlapping with the exposure period, ensuring that observed variations were mostly attributable to the post-transplantation exposure on the CO_2-enriched volcanically active area, while minimizing any variations caused by age differences, such as hyphal thickening in older portions and greater abundance of algal cells in younger portions (Godinho et al., 2009).

The tips were immediately placed on histological cassettes for 48 h of fixation in formalin-acetic acid-alcohol 50 (FAA 50) (Johansen, 1940), followed by dehydration in ascending concentrations of ethanol (70%; 96% I; 96% II; 100%), for one hour each, then by clearing in ascending concentrations of xylene (E3:X1; E1:X1; E1:X3), for 30 min each, and two consecutive immersions in pure xylene, also for 30 min each. Finally, the tips were immersed in a saturated solution of xylol-paraffine, at 37 °C for 12 h, previously to 4 h of paraffin embedding. Solid paraffin blocks were obtained by holding the lichen tips standing upright for transversal cross-sectioning on a rotary microtome. Permanent histological slides were obtained by staining serial cross-sections of 6 μm thickness with toluidine blue (TB), as done by Barbosa, Machado, & Marcelli, (2009) for *Parmeliaceae* lichens, and mounting on DPX medium. A decent metachromatic effect, especially for a good contrast of algal cells, was produced with both 0.1% TB for 20 min and 1% TB for 15 s. In the end, per transplant bag, a maximum of 10 individual and structurally well-preserved histological cross-sections were chosen for further light microscopy observation and histomorphometric measurements. Additionally, a composite sample was obtained from each transplant bag and analyzed for nitrogen content. The results for N determination are in Supplementary Material (Table S1).

2.5. Light microscopy observation of histomorphometric parameters

U. rubicunda lichen thalli have a heteromerous structure with radial organization of layers around a central cord of longitudinally orientated and densely agglutinated fungal hyphae (Ivanovic et al., 2013) (Fig. 2B). The relative volumetric density and the thickness of each thalline layer were determined on the selected histological cross-sections, namely the cortex (C), medulla (including algal cells) (M) and central cord (CC). These layers were further grouped as functional layers, those more closely interfacing with the air and where fungal and algal partnership occurs, (C) and (M), versus the primarily supporting layer of hyphal mass (CC). The M168 Weibel Multipurpose Test System stereology method (Weibel, 1979) was used to obtain the relative volumetric density of C, M and CC at 250 × magnification (Fig. 2C) on a light microscope. Photomicrographs of the same histological cross-sections were taken under a Leica photomicroscope at 200 × magnification. The thicknesses of C, M and the shortest diameter of the CC were measured with the aid of image analysis software Image-Pro® Plus V5.0 (Media Cybernetics, 2004) (Fig. 2D).

The measurements were made on well-preserved zones, clear of artificial hollows or breaks, where continuity is evident from the C down to the CC. A separate gonidial layer was not considered because, in cross-sectional observations, algal cells tend to occur aggregated in pockets, which are very intertwined with the medullar hyphae, rather than forming a well delimited continuous layer. Hence, another methodology was developed for determining the relative abundance of algal cells on the same photomicrographs with high precision. Using layered image editing software paint.NET, V4.2.4 (dotPDN LLC, 2019), a rectangle of 130 μm length by 100 μm width was superimposed over the thalli zone with visibly greatest presence of algal cells, with the top length of the rectangle overlapping with the outer margin of the cortex. Then, the area occupied by algal cells within was obtained and divided by the total area of the rectangle, providing a relative percentage of algal occupation in the thalli (Fig. 2E).

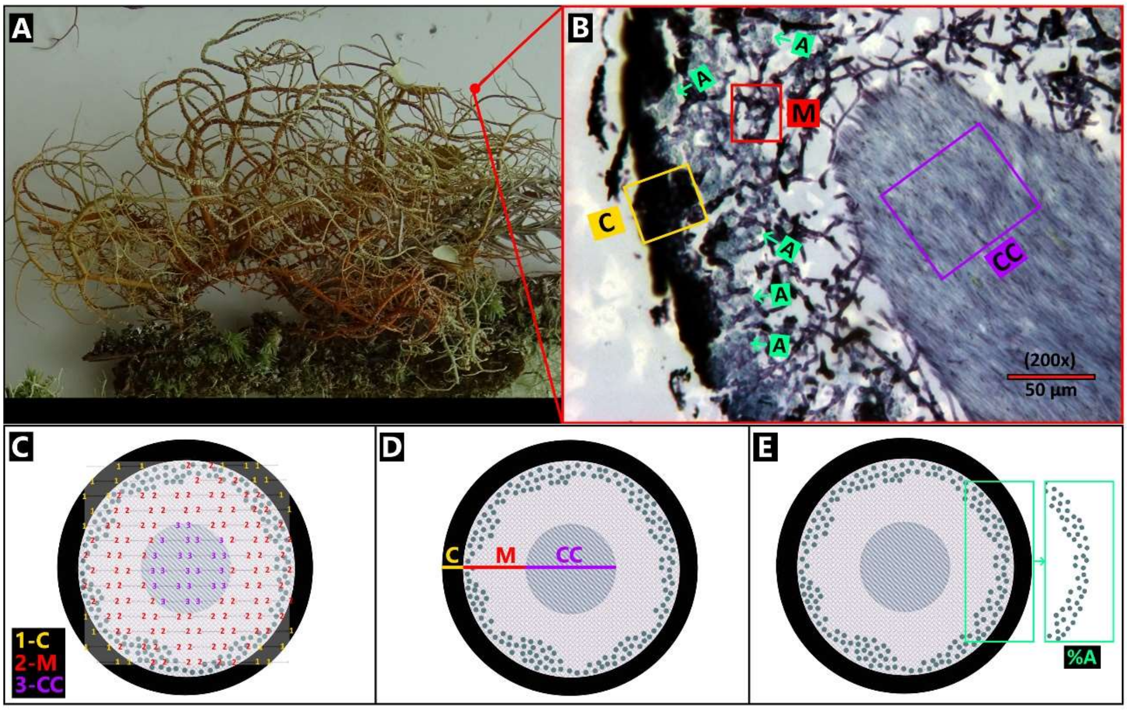

Figure 2. (A) Habitus picture of *Usnea rubicunda* natively occurring in the reference site and used for transplantation to Furnas volcano. (B) Exemplary histological cross-section of the heteromerous *Usnea rubicunda* thalli, stained with toluidine blue for metachromatic effect under light microscopy observation at 200 x magnification, showcasing the three thalline layers, cortex [C], medulla [M] and central cord [CC], as well as green-algae cells [A] (*Trebouxia* sp.). (C) Diagram illustrating the determination of the relative volumetric density of each layer on histological cross-sections. (D) Diagram illustrating the determination of the thicknesses of each layer on histological cross-sections. (E) Diagram illustrating the determination of the relative percentage of algal occupation (%A) within a constant rectangular area placed over the outermost zone of the thallus. See section 2.5 for details.

2.6. Statistical analysis

One measurement was made for each histomorphometric parameter and relative abundance of algal cells per histological cross-section. Student's *t*-test was used to compare means between Furnas group and reference group. Priorly, extreme outlier values of all variables were detected and removed using the IQR (inter-quartile range) rule with a multiplier of 3. Statistical analysis was done with IBM SPSS Statistics V25 (IBM Corp., 2017) and the level of statistical significance was set at $p \leq 0.05$.

3. Results

A total of 326 Furnas and 74 reference *U. rubicunda* thalli cross-sections were considered. The mean values of the two groups for each histomorphometric parameter are shown in Table 1. Furnas samples were overall significantly thicker than reference samples (t (114.676) = 5.582, $p < 0.001$), mostly due to significantly increased thickness, in absolute values, of the medulla (t(107.557) = 2.315, $p < 0.05$) and central cord layers (t(118.462) = 5.633, $p < 0.001$) (Table 1). Even though all layers increased in thickness on Furnas lichens, the cortex (t(98.597) = -2.584, $p < 0.05$) and medulla present a relative decrease of the volumetric density contrasted with the significantly greater relative volumetric density of the central cord (t(136.765) = 6.854, $p < 0.001$) (Table 1).

Table 1. Mean values (± standard error of mean) of the histomorphometric parameters observed in cross-sections of lichen thalli from Furnas transplants and reference site, namely the relative volumetric density (RVD) and thicknesses of each thalline layer (in μm) of the Cortex (C), Medulla (M) and Central cord (CC).

	N	Furnas transplants	N	Reference samples
C (RVD)	326	0.24 ± 0.01 *	74	0.27 ± 0.01 *
M (RVD)	326	0.44 ± 0.01	74	0.46 ± 0.01
CC (RVD)	325	0.32 ± 0.01 ***	73	0.26 ± 0.01 ***
C (μm)	326	31.02 ± 0.48	74	29.21 ± 0.93
M (μm)	324	70.71 ± 1.05 *	74	65.01 ± 2.23 *
C+M (μm)	324	101.75 ± 1.13 **	74	94.21 ± 2.46 **
CC (μm)	325	149.86 ± 1.64 ***	73	130.52 ± 3.02 ***
Total thickness (μm)	325	252.03 ± 2.32 ***	73	224.05 ± 4.44 ***

Student *t*-test, significantly different at:
* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

These results show that, during the exposure period, the symbiotic partnership of Furnas transplanted *U. rubicunda* was able to produce significantly more biomass than reference counterparts, predominantly in the form of fungal hyphal tissue in the central cord, given that Furnas thalli possess, in volumetric terms, a significantly lesser proportion of the functional layers (C + M) versus a significantly greater proportion of the structural central cord (Table 1). In terms of thickness, thalli cross-sections were increased on average by as much as 12.5% ± 1.0%, with central cord thicker by 14.8% ± 1.3% and cortex with medulla by 8.0% ± 1.2% (Figure 3). This is despite the fact that Furnas lichens have a significantly lower percentage of algal occupation (9.741 ± 0.32) in comparison with reference (12.853 ± 0.80) ($t(85.991) = -3.753$, $p < 0.001$) (Figure 4). Contrarily, reference lichens were unable to produce as much fungal biomass over six months, despite having a significantly greater percentage of the algal occupation, indicating that the relative abundance of algal cells was not a limiting factor for the productivity of either group. Algal cells occupy a relatively smaller area in the enlarged Furnas thalli cross-sections, yet these were able to generate and sustain the additional volume of fungal biomass produced above that of reference lichens. This indicates greater efficiency of the algal cells in fueling biomass productivity over the time of the experiment, as lichen productivity involves the fixation of CO_2 from the air by the photobiont to produce carbohydrates which are transferred to the fungal partner (mycobiont) in a profitable pace to compensate for maintenance respiration and provide for building new fungal biomass (Palmqvist, Dahlman, Jonsson, & Nash, 2008; Seminara et al., 2018).

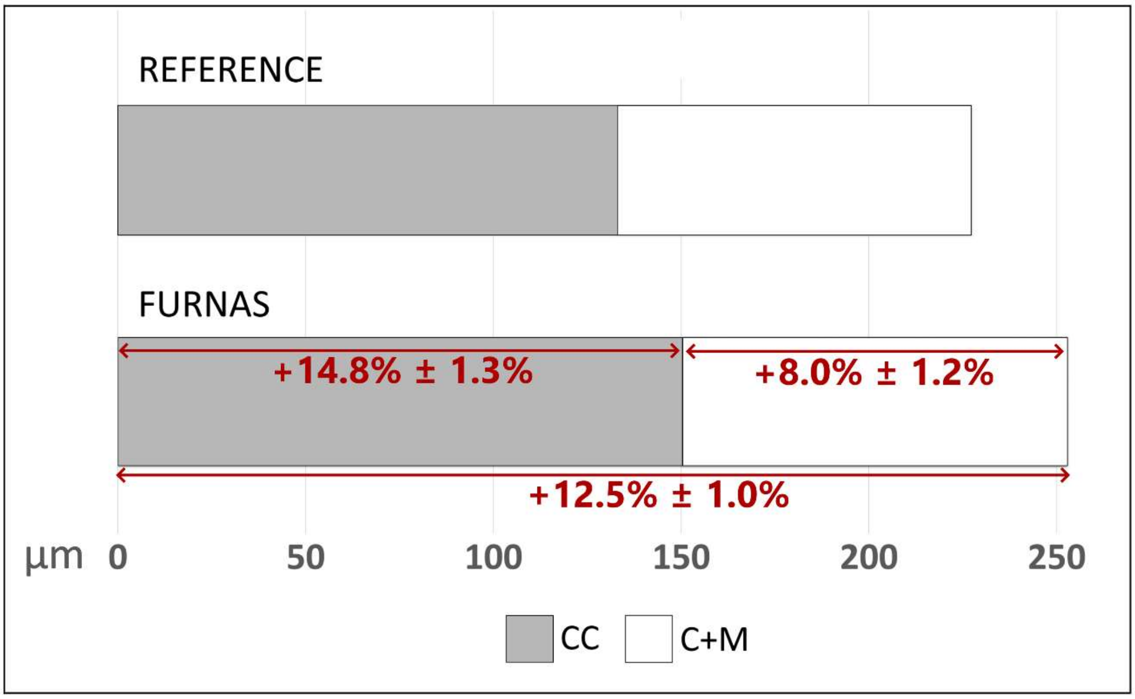

Figure 3. Average thickness increases of cortex with medulla (C + M) and central cord (CC) layers in *Usnea rubicunda* thalli cross-sections of Furnas transplants in comparison with reference. The percentage variations pertain to the differences in mean values of thickness (µm), whose comparisons and statistical significance are presented in Table 1.

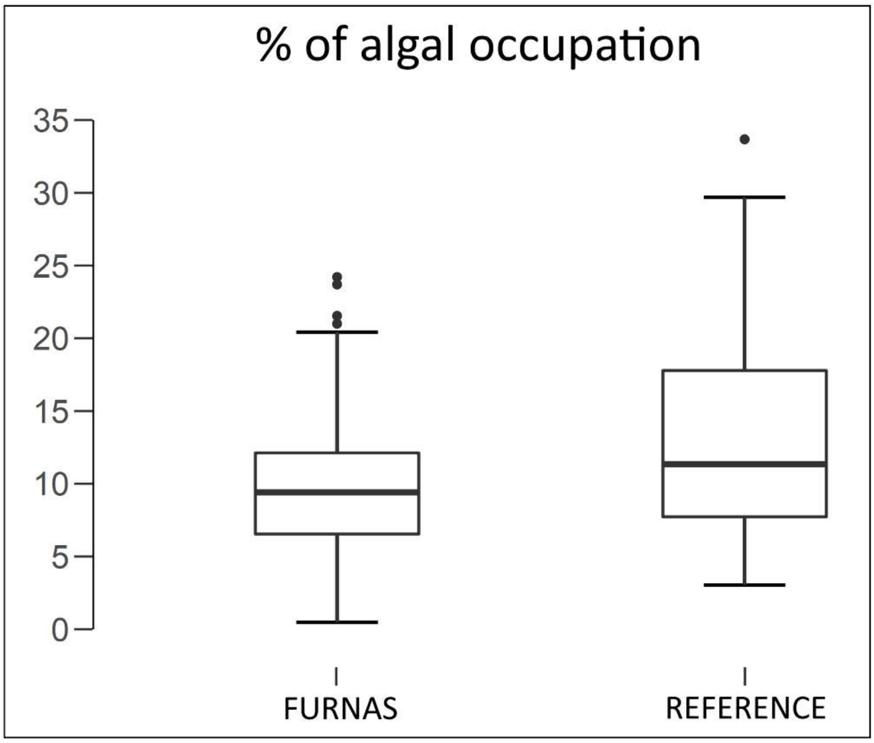

Figure 4. Boxplot diagram showing the distribution of the relative percentage of algal occupation (%A) in *Usnea rubicunda* thalli cross-sections of Furnas transplants (9.741 ± 0.32, s.d. = 4.206) in comparison with reference (12.853 ± 0.80, s.d. = 6.844). Means of the two groups are significantly different $p < 0.001$ (Student *t*-test). For the determination of the algal occupation, see details in Figure 2E.

4. Discussion

Overall, the results of this field study suggest that the symbiotic partnership was not reaching its productivity potential to the fullest while lichens remained in the reference site but benefitted of a biomass productivity boost after transplantation and exposure at the volcanic area. Such effect can be attributed to the greater availability of airborne CO_2 for the photobiont on Furnas caldera. As per the study's design, detailed in section 2, the choice of Furnas caldera ensured a steady source of hydrothermal CO_2 emissions in similar macroclimatic conditions and within a rural setting where, like the reference site, anthropogenic influence and pollution is low and otherwise just as favorable to the occurrence and growth of a sensitive fruticose species such as *U. rubicunda*. Furthermore, though Furnas transplants were distributed across multiple locations intra-caldera, no significant patterns of variation were found for the histomorphometric parameters between Furnas samples, hence treated together as a group, indicating that the observed structural response is relevant at the level of the whole caldera. This is consistent with the spatially diffusive nature of soil degassing emissions, which dominate the total CO_2 budget of Furnas volcano (Pedone et al., 2015; Viveiros et al., 2010) and should cause CO_2 levels to be increased relatively evenly in the air at transplant height over the whole caldera. Therefore, on a general level, Furnas transplants are expected to have enjoyed a more CO_2 enriched air mixture than reference samples.

Accordingly, the enlargement of the thalline layers observed in Furnas group reflects a rather beneficial, time-integrated response of *U. rubicunda* to the transplantation into the CO_2 enriched volcanic environment, supporting the view that lichen primary productivity is amplified by increased atmospheric CO_2 levels. However, for this thriving response to CO_2 to be sustained, it must involve the favorable conjugation of other limiting factors affecting lichen productivity. Firstly, the constantly high relative air humidity under a temperate climate in our location should slow down thalli drying between moistening episodes by dew, fog or rain, allowing longer lasting moderate water contents leading to profitable photosynthetic rates (Lange & Green, 1996).

Besides, no significant differences were found in thallus nitrogen content between Furnas transplants and reference samples (see Supplementary Material). Low nitrogen availability may trigger photosynthetic acclimation of lichenized algae following prolonged exposure to elevated CO_2 (Balaguer et al., 1996). In the field study conducted by Balaguer et al., (1999), the foliose lichen *Flavoparmelia caperata*, occurring *in-situ* near a geothermal vent, under prolonged exposure to elevated CO_2, showed similar thallus nitrogen content and did not exhibit down-regulation of photosynthesis when compared to thalli at ambient CO_2. However, despite the absence of photosynthetic acclimation, their results showed that biomass production of this foliose lichen in the vicinity of the geothermal vent was not promoted by increased CO_2. We propose that using transplants rather than *in-situ* lichens, within a defined exposure period, delivers a clearer experimental design to ascertain the effect of elevated CO_2 on lichen productivity in field conditions, by ensuring that all samples were originally accustomed to ambient CO_2 concentrations. This distinct approach lessens potential genetic and physiological divergence of species strands naturally occurring with elevated CO_2, as well as avoids limitations imposed by the phorophyte on lichen development (Balaguer et al., 1999).

Also, all histomorphometric measurements were restricted to the thalli elongation zones, the primary drivers of growth where carbon influx is greatest (Seminara et al., 2018). Parameters applied at the level of the whole lichen thalli could overlook the structural variations in relation to elevated CO_2 at these thalli zones of maximum development. Moreover, a conceivable stunting influence of other pollutants of volcanogenic origin on lichen productivity is even less applicable in our study. Sampling sites were spread to cover homogeneously the area within Furnas caldera, encompassing the gradient of soil diffuse CO_2 degassing areas (Viveiros et al., 2010) rather than focusing on the vicinity of the main surface emanations, in our case the Furnas fumarolic fields, which are localized sources of H_2S and other volcanogenic gases and aerosols besides CO_2 (Ferreira & Oskarsson, 1999; Pedone et al., 2015). Balaguer et al., (1999) also pointed out that a lower rate of light saturated CO_2

assimilation, observed on the lichens at high external CO_2, may have reduced potential benefits of long-term CO_2 enrichment. Contrarily, Nash (1996) reports a sustained increase in photosynthetic assimilation in two other lichen species at even higher CO_2 concentrations than those measured by Balaguer et al., (1999). Evidence from Free-air CO_2 enrichment (FACE) experiments in plants also substantiate that photosynthetic carbon uptake is greater with elevated CO_2, resulting in increased carbon gain despite photosynthetic acclimation, whose extent varies with genetic, experimental and environmental factors (Leakey et al., 2009).

Thus, it can be inferred that different lichen species and growth forms might not respond similarly to CO_2 enrichment, even if sharing a CO_2 sensitive green-algal photobiont (*Trebouxia*). Broad predictions concerning the effects of rising CO_2 concentrations over the vast diversity of lichen flora should be taken cautiously, as conclusions from field studies must consider the disparity of lichen species, experimental designs and local conditions. In this regard, the results of our study suggest that *U. rubicunda* is a good bioindicator lichen species with sensitivity to atmospheric CO_2 variations, therefore suitable for CO_2 biomonitoring field studies in natural environments.

The histomorphometric approach applied to lichens in this study was demonstrated as a valid methodology to quantify significant structural alterations in the heteromerous thalli of a CO_2 sensitive green-algae lichen. These thalli alterations constitute suitable response biomarkers in *U. rubicunda* with potential to indicate airborne CO_2 levels. Previously, the organization and structure of lichen thalli have been shown to be responsive to both environmental and anthropogenic factors (Pintado, Valladares, & Sancho, 1997). Changes in the thicknesses of thalline layers are among the first lichen reactions to air pollution (Osyczka et al., 2018; Otnyukova, 2007). Furthermore, histomorphometric parameters do not reflect instantaneously occurring processes, such as photosynthetic rate, but reflect the overall time-integrated physiological performance of lichens. Particularly in field studies, time-integration is fundamental to account for the combination of multiple environmental stressors and their temporal variability. For instance, as lichens require enough moisture to be photosynthetically active (Nash, 1996), their productivity is dependent on their water content (Lange & Green, 1996), which can oscillate throughout time. Thus, these structural biomarkers are useful to track and integrate the influence of time-mediated environmental disturbances.

5. Conclusion

The histomorphometric parameters applied in this study confirmed the initial hypothesis that *U. rubicunda* thalli undergo significant structural alterations as primary responses to the transplantation from a reference site to an actively degassing volcanic environment. In general, the greater availability of airborne CO_2 in the hydrothermal area promoted lichen biomass productivity under non-limiting climatic conditions, as displayed by thicker thalli and a proportional increase in the relative volumetric density of the fungal central cord, despite the decreased relative percentage of algal occupation, indicating a greater capability of algal cells to sustain more fungal biomass with higher CO_2 levels. These results demonstrate that histomorphometric measurements constitute time-integrated response biomarkers in *U. rubicunda*, carrying potentially interesting application in further research aiming to understand how lichens adapt to pressing environmental changes over time, such as the global rise in atmospheric CO_2, and how lichens might be used as suitable bioindicators of increased CO_2 atmospheric concentrations in natural environments.

Credit author statement

Filipe Bernardo: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. Tânia Rocha: Methodology, Validation, Formal analysis, Investigation, Writing - review & editing, Visualization. Cristina Branquinho: Writing - review & editing, Supervision, Funding acquisition. Patricia Garcia: Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. Armindo Rodrigues: Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix – Supplementary material

Table S1. Descriptive statistics for %N. Mean values are not significantly different between Furnas transplants and reference samples ($\chi^2 = 0.517$, df = 1, $p = 0.472$) (Kruskal-Wallis H test).

	%N					
	N	Max.	Min.	Range	Mean	Std. Dev.
Furnas transplants	36	1.63%	0.74%	0.89%	1.11%	0.22%
Reference samples	6	1.23%	1.07%	0.16%	1.16%	0.05%

Methodology for %N determination

A composite sample of *Usnea rubicunda* from each Furnas transplant and reference sample was obtained after cleaning of debris and removal of visibly moldy or damaged portions. The selected lichen material was oven dried at 40 °C for 72 h. No preliminary water cleansing was made to prevent washing away of a significant elemental load. Around 10% of the total weight was lost after oven drying. Homogenization of dried lichen material into powder was done by cutting and manual grinding with mortar and pestle. Elemental quantification analysis was done at the Stable Isotopes and Instrumental Analysis Facility (SIIAF), at the Faculdade de Ciências, Universidade de Lisboa – Portugal, on a EuroEA (EuroVector Italy) elemental analyzer for online sample preparation by Dumas combustion. The major mass signals of N were used to calculate a percentage of N content (%N) in samples a using an elemental composition reference material, WFS OAS for N with 1.47% N (Elemental Microanalysis, UK).

CHAPTER 4

Conclusion

Fulfilling the thesis rationale and main objective

The development of this thesis was grounded on a focused research effort proposing and demonstrating the application of biomonitoring tools dealing with concrete and relevant sources of natural and anthropogenic atmospheric pollution coexisting in the distinctive environmental setting of volcanic islands. Selected lichen species, either transplanted or naturally occurring, were collected after exposure to hydrothermal degassing at an active volcano, as well as at different sites pertaining to three main land uses, comprising agricultural and artificial areas in addition to forest areas, thus representing the ongoing occurrence air pollution originated from both volcanogenic and anthropogenic emissions.

Overall, the studies of this thesis clearly display the value of applying biomonitoring methods, based on lichens as well-established bioindicators of air pollution, under more focused and properly designed environmental assessments targeting relevant and localized sources or activities potentially contaminating and threatening environmental and human health. For instance, at the regional level of the Azores islands, the case study where the work was developed, these methods demonstrated validity to complement conventional air quality monitoring routines, presently limited to three (rural and urban) background monitoring stations (two in São Miguel), extending it beyond the current general assessment of air quality, mostly grounded on verification of compliance of atmospheric background concentrations with legislation limits. However, air quality might not be uniform throughout the entire territory all the time, as the levels of air pollutants and degree of exposure are time and distance-dependent relative to the location and intermittency of release from the emission sources. Thus, the logistic flexibility of biomonitoring campaigns, allowing better spatial representativity coupled with the time integration provided by biomonitors, is well suited to address more circumscribed scenarios of known or suspected air pollution in the Azores. The increased spatial resolution provided by biomonitoring data through these tendentially cheaper and finer nature-based solutions allows for greater precision in territorial planning, leading to more effective mitigating measures against air pollution.

For instance, the threat posed by the emissions of volcanogenic air pollutants, as evidenced by the adverse health effects observed in human populations following acute overexposures or chronic exposure in volcanically active areas, affects particularly the inhabitants of Furnas and Ribeira Quente villages, in the vicinity of Furnas volcano, but not the rest of the population of the island. This configures an exemplary scenario of enduring poorer air quality, in comparison with the rest of the territory, potentially affecting human health, that is not directly covered by the background monitoring stations, the closest one located several kilometers apart. However, with the use of biomonitors *in-situ* at Furnas caldera, it was possible to design a dense sampling grid, stratified by the local soil CO_2 flux degassing gradient, allowing the determination of the risk areas of chronic exposure to outdoors volcanogenic emissions for Furnas inhabitants with high spatial resolution and time integration, even under the influence of uncontrolled field conditions such as atmospheric fluctuations. The implementation of such biomonitoring-based exposure surveys, specifically dedicated to the areas under volcanic influence, especially when conjugated with indoor CO_2 and ^{222}Rn surveys, should allow a much tighter correlation with data on health outcomes of the local inhabitants than otherwise possible with sparser air pollution data from background monitoring stations, providing solider evidence to estimate the impacts of volcanic air pollution, guide adequate protective measures and decide where to establish dwelling places. In this regard, this thesis introduces novelty considering that biomonitoring studies, applying lichen transplants in dealing with eruptive and particularly non-eruptive volcanic activity, remain relatively rare (compared with anthropogenic emissions), despite being one of the major natural sources of toxic air pollutants, whose impact in inducing adverse health outcomes following chronic exposure is poorly known (Rodrigues & Garcia, 2015).

Furthermore, the elemental profile of native lichens resident at each of the three primary land uses of São Miguel, expressing a long-term exposure, over the extended lifetime of these slow-growing bioindicators, to the atmospheric composition characteristic of each land use, displayed the ongoing decline of the pristine excellency of air quality in this island due to the emissions resulting from intensifying agricultural and urban activities. Such decline has not been as evident judging from the last decade of air quality monitoring reports in the region, appraising air quality as consistently "good" (DRA, 2019) by not exceeding allowable legislation limits. Yet, the application of biomonitoring metrics uncovered a significant anthropogenic disturbance, manifested by the assimilation of air pollutants along the gradient from the less impacted forest areas to the more impacted agricultural and artificial areas, demonstrating that air quality is not uniformly as good. This presents another distinct environmental scenario, in addition to Furnas volcano, where the representativity of the sparse network of monitoring stations is lacking to address the entire spectrum of challenges posed by air pollution in the Azores, greatly benefitting from complementation with biomonitoring tools and more refined environmental assessments. A notorious example of such a challenge is the significant airborne nitrogen pollution from intensifying agriculture detected by native lichens. The results showed a bioaccumulation of nitrogen with an isotopic signature corresponding to readily reactive nitrogen in its reduced ammoniacal form, whose excess has cascading negative effects in the environment (Pinho et al., 2017). Until now, past studies dealing with nitrogen contamination in the Azores were mostly limited to eutrophication of surface water bodies and underground water reserves via runoff and infiltration, whereas this thesis brought a novel focus upon the major contribution of airborne pathways in the dispersal of nitrogen from agricultural emissions into the environment. The suitability of lichens as biomonitors of nitrogen deposition warrants their use *in tandem* with conventional monitoring for adequate management of this serious contamination problem.

The remote oceanic location of insular regions like the Azores, moreover distant from major hotspots of air pollution, is favorable to a greater regeneration capacity of the atmosphere to increasing emission loads (DRA, 2019). Nevertheless, island terrestrial ecosystems are of the most fragile and vulnerable of the planet to environmental disrupting factors (Haase and Maier, 2021), given their context of remoteness and isolation, naturally shaped by a low level of disturbance (Fernandes et al., 2015). Growing levels of disturbance, manageable in mainland territories, meet a lower threshold of tolerance in such particularly fragile insular regions, thus more easily threatening their ecosystem services that substantially contribute to the local economy and provide crucial contributions to appeal to the nature-based touristic sector (Sieber et al., 2018). Therefore, ensuring the preservation of excellent air quality in these regions requires that the patterns of air quality be held to high standards, not overlooking the relatively small-scale human activities, whose impact would be less perceptible in a mainland context. The case study employed in this thesis evidenced that the elemental profile of native lichens from agricultural and artificial land uses already present a contaminated condition significantly departing from the more pristine forest areas of São Miguel Island. However, air quality deterioration is still not wholly evident by exceedance of legislation limits of air pollutants, the only standard so far applied in the Azores. In line with the EU strategy for outermost regions of Europe (European Commission, 2017; Haase and Maier, 2021), and according to the revealed by this thesis, the implementation of continuous biomonitoring surveillance of air quality is commendable towards a greener and more sustainable development at susceptible insular regions.

Limitations and future research opportunities

Despite reaching its main purpose of demonstrating the application of biomonitoring tools of air pollution at the regional level, further research opportunities are left open, deserving consideration in the design of future subsequent studies. Most notably, it is of the utmost interest to explore a correlation between the data gathered with lichens as biomonitoring tools and data on adverse effects on human health, giving due sequence to the second study of this thesis, dealing with chronic exposure to hydrothermal volcanic emissions for one of the Azorean populations most endangered by air pollution. Since the final output of this study consisted in a map of the risk areas of chronic exposure, based on residential areas as proxy of ongoing human presence, the establishment of a link between each risk level of chronic exposure and the incidence of acute or chronic adverse respiratory conditions will allow the evaluation of the efficacy of the spatial model in predicting the development of health outcomes. The purpose is similar to the notorious study by Cislaghi and Nimis (1997), which related mortality by lung cancer in young men at Veneto region, Italy, but with lichen diversity metrics instead. By taking advantage of this new spatial model to stratify a future sampling design in accordance with the risk levels proposed, additionally accounting for both indoor and outdoor exposure routes, a more robust approach is possible to conceive a groundwork study involving human biomonitoring at Furnas volcano. Further lichen biomonitoring studies should also be dedicated to find other suitable tracers of the dispersal of airborne volcanic emissions devoid of sulfurous content, thus adapting the model to volcanic areas where its inviable to rely on the $\delta^{34}S$ signature .

Considering that lichens are "sentinel" organisms of air pollution, owing to their deep reliance on atmospheric exchange, they are of the first and most affected, providing more immediate information. However, an integrative approach involving the combination of metrics in multiple bioindicator organisms is necessary to ascertain the full extent of the impacts associated to airborne emissions, downstream of lichens, up to the whole ecosystem, for instance by analyzing vascular plants or wild mice living along the same environmental gradients or around the same pollution sources that were sampled with lichens. Such expanded surveys to consider in the future, linking with other metrics outside the lichens themselves, will tie more adequately with the complete definition of air quality, which goes beyond determining the degree of contamination of the atmosphere (and using lichens merely as passive bioaccumulators) to unfolding the corresponding burden of effects over living systems in interaction with it (Garty, 2001), further filling the gap in interpreting atmospheric concentrations from conventional monitoring at the level of the biosphere.

The results of the third study of this thesis justify follow-up research involving physiological or metabolic parameters in lichens, namely photosynthesis and respiration rates or F_v/F_m ratio. While the approach followed was primarily intended to propose histomorphometric parameters to measure structural alterations as time-integrated response biomarkers of CO_2 levels in natural environments, a combination with metrics of lichen physiological performance would further validate the observed effect of increased biomass productivity. This is because the photosynthetic performance behind the productivity gain was not directly measured in the lichens but rather indirectly detected by the structural alterations, indicating fungal biomass increment, in the transplanted samples subjected to a prolonged exposure to the greater availability of airborne CO_2 at Furnas volcano caldera. As an aside, further studies employing lichen transplantation should also preferably include snapshots for sampling retrieval at intermediate periods to track fluctuations between the starting and the ending of the exposure period.

At last, the first study of this thesis was based on a simplified sampling design by the primary land use gradient of São Miguel Island, which agglomerated sampling sites for collection of native lichens without further distinction between separate activities occurring within the same land use. Thus, it was not adequate to differentiate and compare the influence of farming with that of livestock within agricultural land use, and likewise, within artificial land use, the impact associated to industrial facilities with that of residential-like urban areas. However, since this general assessment has clearly revealed a significant degree of anthropogenic disturbance in artificial and agricultural areas, the precedent is fully set to conceive and carry out multiple follow-up studies dedicated to weigh the relative impact of various types of anthropogenic activities, for instance comparing emissions associated to different farming systems, such as traditional, organic, and conventional farming, or comparing livestock production systems of varying intensity.

Final considerations

This thesis, planned and proposed under the framework of the Strategy of Research and Innovation for Intelligent Specialization of the Autonomous Region of the Azores (RIS3-Açores), was a successful effort dedicated to bringing greater attention to local companies, research entities, public entities, and society in general, regarding the subject of air pollution. It also contributed to equipping the region with effective biomonitoring tools to improve air quality monitoring procedures, ensuring adequate surveillance of the natural patrimony of the Azores and motivating a sustainable exploitation of the touristic assets of the Azorean "green environment", as preconized in one of the three main thematic lines of RIS3.

However, it is also worth reminding that the threats posed by air pollution are not only a domestic issue, limited at the regional scale, but are also a global challenge. Even a remote location is not immune to the world's increasing rate of air pollution since it is not restricted by geographical boundaries. Through long-range transport, acidifying gases like SO_x and NO_x, ground level ozone, particulate matter, or persistent organic pollutants, can affect air quality at distant locations, which has led to the framing of the Geneva convention on Long-Range Transboundary Air Pollution, establishing a committed cooperation between several countries for the prevention, reduction, and monitoring of these atmospheric pollutants (Byrne, 2017). Moreover, despite having a relatively tiny contribution to worldwide emissions of greenhouse gases, insular regions like the Azores are the most vulnerable to detrimental shifts induced by climate change, further aggravated by their synergistic interaction with local human-induced threats (Veron et al., 2019).

Therefore, as especially interested parties, it is paramount that the Azores and other insular regions worldwide are actively engaged and involved in research, policymaking and action-taking pertaining to air quality and climate change, as the outcome of global efforts will eventually find its corresponding repercussions at the local level. The concerns and challenges of these small and vulnerable insular territories must be properly voiced and acknowledged in the international stage, amidst those with greater power and influence but perhaps also more predisposed to inaction. In this regard, it is desirable that insular territories cooperate more closely in exchanging information and scientific innovation, carrying forward the common goals of environmental sustainability and preservation. As it presently stands, the outlook is quite grim, seeing that 91% of the global population inhabits places where WHO air quality guidelines are not met (WHO, 2018), entailing a distressing burden of disease and premature death (Khomenko et al., 2021). Air pollution is projected to become the top cause of environmentally related deaths by 2050 (OECD, 2012). But it is not yet too late if the current generation commits to change for the sake of future generations. The Azores can lead by example and take a very active role in reverting this scenario.

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Biomonitor air quality of the Azorean green environment

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