“Advances in air quality biomonitoring with the terrestrial moss ***Pseudoscleropodium purum***”

Zulema Varela Río
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“Advances in air quality biomonitoring with the terrestrial moss ***Pseudoscleropodium purum***”

Memoria que presenta
Zulema Varela Río
para optar al Grado de Doctora en Biología

Fdo: Zulema Varela Río

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CERTIFICAN:

Que la presente memoria titulada **"Advances in air quality biomonitoring with the terrestrial moss *Pseudoscleropodium purum*"** presentada por **Dña. Zulema Varela Río** para optar al **Grado de Doctora en Biología** ha sido realizada bajo nuestra dirección en el Área de Ecología del Departamento de Biología Celular y Ecología de la Universidad de Santiago de Compostela. Asimismo consideran que dicho trabajo está en condiciones de ser defendido, como Tesis Doctoral, ante el Tribunal correspondiente.

Y para que así conste, firmamos la presente en Santiago de Compostela 9 de Marzo de 2014.

Fdo: Jesús R. Aboal Viñas

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A Susi, Luis y Rebeca


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“Be less curious about people and more curious
about ideas”

Marie Curie.

“The secret of life is honesty and fair dealing.
If you can fake that, you’ve got it made!”

Groucho Marx.


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GENERAL INTRODUCTION AND OBJECTIVES


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INTRODUCTION

Terrestrial bryophytes are widely used to estimate the levels of contaminants in the atmosphere. The so-called “moss biomonitoring technique” was developed in the Nordic countries at the end of the 1960s (Rühling and Tyler, 1968; Tyler and Rühling 1970) as a means of studying the atmospheric deposition of heavy metals. Bryophytes are often considered as ideal organisms for use as biomonitors because of particular characteristics that confer them with a high cation exchange capacity, which enables them to capture and accumulate contaminants from atmospheric deposition. In addition, they can also retain particles (which become strongly bound to their tissues), they are widely distributed in various types of habitats, and they can be easily and inexpensively maintained in the laboratory. Use of the technique has spread to other European countries, and mosses have been used to estimate the levels of different types of contaminants: organic compounds (*e.g.* Knulst *et al.*, 1995; Lead *et al.*, 1996; Orlinski, 2002; Gerdol *et al.*, 2006; Carballeira *et al.*, 2006), radioactive elements (*e.g.* Sawidis and Heinrich, 1992; Triulzi *et al.*, 1996), non metals, such as nitrogen (*e.g.* Harmens et al., 2011), and halogens, such as fluorine (*e.g.* Real *et al.*, 2003).

The success of the technique has led to the creation by the United Nation Economic Commission for Europe (UNECE) of a European monitoring programme (The International Cooperative Programme on Effects of Air Pollution on Natural Vegetation and Crops: ICP Vegetation), which uses bryophytes to monitor the atmospheric deposition of heavy metals, nitrogen and persistent organic pollutants (POPs). The aims of the biomonitoring network are to obtain qualitative and quantitative measures of the atmospheric deposition of heavy metals in Europe, to locate the sources of emission of these contaminants, to construct maps of regional patterns of contamination and to carry out retrospective comparisons of the results of different surveys.

Since 1995, the Ecotoxicology research group at the University of Santiago de Compostela has carried out numerous studies aimed at optimizing the technique of biomonitoring air quality using terrestrial mosses, mainly *Pseudoscleropodium purum* (Hedw.) M. Fleisch. This bryophyte is widely distributed in temperature regions in the northern hemisphere and is particularly abundant in Galicia. *Pseudoscleropodium purum* is one of the easiest mosses to recognize in the field and handle in the laboratory. It is a robust, green or yellow-green plant with more or less regularly pinnate shoots, of 10 cm or longer, and relatively short branches (the shoots are therefore feather-like). The leaves are erect, loosely appressed and overlapping, only a little longer than wide, and deeply concave. This gives the typically 2 mm wide shoots a stout, fat appearance. The leaves are about 2 mm long, broadly rounded or broadly pointed, and their most distinctive feature is the presence of a small, curved point at the tip. At the tip of the stem and new branches, the crowded points of the leaves protrude in the form of a miniature crown (older branch tips may become more attenuated). The leaves have a single nerve. Branch leaves are similar to the stem leaves, but a little smaller. Capsules are rare.

Despite the concerted research effort of the group (more than 50 international publications in the last 15 years), further research on some aspects (methodological and ecophysiological) of the technique is still required to optimize the use of *P. purum* as a biomonitor. Most importantly, there is not yet a standardized scientifically-based protocol for applying the moss biomonitoring technique. The protocol first proposed by Rühling (1989) has been minimally modified to produce the current version (Harmens et al., 2005). Delimitation of the sampling sites is one of the key factors involved in applying the technique, as this will greatly affect the results obtained. However, the optimal dimensions of the sampling sites have not yet been established scientifically. In Chapter I of this doctoral thesis, I address the need to standardize the method, beginning by establishing the optimal size of sampling sites for use of the moss *P. purum* as a biomonitor.

The next important step is to test the suitability of *P. purum* for biomonitoring other types of contaminants that are not usually studied in moss, but that are relevant to air quality, such as nitrogen (which I consider in Chapter II). Nitrogen emissions have increased in the last few decades as a result of the increased demand for food and energy worldwide. Deposition of atmospheric N derived from different anthropogenic activities has thus become the major source of N in some ecosystems. However, studies carried out to date have shown only a weak relationship between the deposition of N and its accumulation in moss tissues. This weak relationship may be explained by regulation of the tissue contents of N in mosses; indeed, the importance of N in bryophyte metabolism has already been identified (*e.g.* Koranda *et al.*, 2007; Arróniz-Crespo *et al.*, 2008). A method involving determination of the relative content of the stable isotopes of nitrogen (^{14}N and ^{15}N) by measuring the $\delta^{15}N$ ratio has recently been proposed (Zechmeister *et al.*, 2008) as an alternative to the biomonitoring method based on measurement of the total concentration of N in mosses.

The increased industrialization of many areas in recent decades has also created the need for a method of characterizing atmospheric depositions that would enable temporal changes in these to be identified and that would also enable different industries to improve their performance in relation to the environment. The industrial sector is the main cause of emissions of heavy metals and metalloids to the atmosphere. Official inventories of emissions, such as the European Pollutant Release and Transfer Register (E-PRTR; http://prtr.ec.europa.eu/) provide information about the contamination emitted by the main industrial plants in the European Union, Island, Norway, Liechtenstein, Serbia and Switzerland. However, most of the available data are estimates rather than direct measurements of emissions. In addition, some contaminants are emitted but not included in the inventory; these are referred to as *false negatives*. In this context, it is important to determine whether biomonitoring with *P. purum* would prevent false negative results for heavy metals and metalloids and thus enable construction of an complem entary inventory (which I consider in Chapter III).

As already mentioned, moss is widely considered as a suitable biomonitor for estimating the atmospheric deposition of heavy metals and metalloids. However, some authors have suggested that moss samples do not always adequately reflect the atmospheric levels of certain elements, probably because of differences in the physicochemical characteristics of the pollutants and in the physicochemical processes in mosses (*e.g.* Aboal *et al.* 2010). The next logical step was therefore to investigate the extent to which the uptake of elements in the moss *P. purum* depends on the physicochemical characteristics of the moss (which I address in Chapter IV). Although moss is used in many biomonitoring studies, the specific physicochemical mechanisms of bioabsorption are less well known than those of other organisms (González and Pokrovsky, 2014). In fact, the possible effects of the physicochemical characteristics of pollutants (*e.g.* amount of contaminant and form of emission) and of the physicochemical processes in mosses (*e.g.* affinity of the contaminants for cation exchange sites, competition between metals/metalloids for cation exchange sites, synergism in contaminant uptake) on the uptake of different atmospheric contaminants have not yet been studied.

Chemical biomonitoring only provides information about the concentration of a contaminant in moss, and therefore alternative means of biomonitoring, *e.g.* by considering physiological parameters, are required. In other words, instead of determining atmospheric contaminants in the biomonitors, the effects that these have on the growth or physiology of the organisms should be evaluated. Such effects have been considered in relatively few studies (Tuba *et al.*, 1997; Tremper *et al.*, 2004; Aboal *et al.*, 2008. In Chapter IV, I report a novel line of investigation used to observe the relationships between successive processes related to the atmospheric contaminants (*e.g.* atmospheric deposition of elements, the concentrations of these in *P. purum*, the response of physiological parameters such as the chlorophyll content index [CCI: Lichtenthaler *et al.*, 1996] and the photosynthetic efficiency index [Fv/Fm: Bolhàr-Nordenkampf *et al.*, 1989]) and how they effect the growth of moss.

However, reference (threshold) values of these physiological parameters have not yet been established in moss (*i.e.* the values obtained in the absence of physiological stress generated by contaminants) to enable correct interpretation of the results obtained. In the final chapter of this thesis (Chapter V), I consider the use of transplants of the moss *P. purum* for establishing threshold values for the CCI and the photochemical reflectance index (CHL) (Fillela *et al.*, 1996) and also the temporal and regional variations in these values. Future research will focus on characterizing and establishing references values of the Fv/Fm ratio, which were not available in the aforementioned study.

OBJECTIVES

1. To propose a method of determining the optimal size of sampling area for application of the biomonitoring technique with the moss *P. purum*, by examining the spatial structure in the concentrations of As, Cd, Cu, K, Hg, Ni, Pb, Se and Zn in samples collected within short distances (<30 m) (Chapter I).

2. To verify the suitability of *P. purum* as a biomonitor of atmospheric contamination, as follows (Chapters II, III and IV):

2.1. By determining whether the total N concentration or the $\delta^{15}N$ ratio in the tissue of this moss species is a good indicator of atmospheric N via confirmation of the following hypotheses: i) that there are no interspecific differences in the concentrations of either total N or $\delta^{15}N$ between the two species of terrestrial moss usually used in monitoring surveys, and ii) that there is no spatial structure in either the total concentrations of N or the concentrations of $\delta^{15}N$ in the surroundings of large scale or small scale sources of N emissions.

2.2. By determining whether the species can characterize the emissions of heavy metals and metalloids in the surroundings of industrial facilities and thus enable the elaboration of an inventory of emissions, through i) identification of any common patterns of emissions for individual industrial sectors, ii) comparison of any such patterns with previously described patterns, and iii) compilation an inventory of the heavy metals and metalloids emitted by the industries considered.

2.3. By examining the extent to which the uptake of elements in mosses depends on the physiochemical characteristics of these plants.

3. To study the extent to which the physiological responses observed in transplanted specimens of *P. purum* enable use of the active biomonitoring technique to characterize the air quality, as follows (Chapter V and VI):

3.1. By determining any relationships between the concentrations of elements in bulk deposition and in moss, physiological traits and growth of the mosses, and by determining whether any such relationships are affected by the duration of exposure and level of contamination.

3.2. By investigating the existence of any seasonal patterns, regional variability, and reference thresholds of Photochemical Reflectance Index (PRI) and the Chlorophyll Content Index (CHL index) in terrestrial moss *P. purum*.


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CHAPTER 01


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Determination of the optimal size of area to be sampled by use of the moss biomonitoring technique

Zulema Varela · J. A. Fernández · J. R. Aboal · C. Real · A. Carballeira

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Abstract The aim of the present study was to determine the optimal size of the sampling area that should be used in the moss biomonitoring technique, in relation to the spatial variation in the concentrations of contaminants determined in samples collected within short distances (<30 m). For this, the spatial structure in the concentrations of various metals and metalloids was explored by use of semivariograms calculated by use of a robust estimator of the variance. In each of the 3 sampling sites considered in the study (one sampled on two occasions), 50 samples of the moss *Pseudoscleropodium purum* were collected and the tissue concentrations of As, Cd, Cu, Hg, K, Ni, Pb, Se and Zn were determined. The results obtained show that in most cases (25 out of 32), there was no spatial structure in the concentrations of the elements and that the semivariograms were subject to the nugget effect. For those elements for which spatial independence or a linear spatial structure was observed, the size of the sampling area did not affect the results, and the dimensions were irrelevant within the range studied. The existence of spatial structure, which can be fitted to a spherical model, enabled a range of autocorrelation to be defined, which corresponded to an area of diameter >16 m. The proposed method must be applied to other species and in other regions, in order to standardize the size of the sampling sites in the moss biomonitoring technique.

Keywords Atmospheric contamination · *Pseudoscleropodium purum* · Semivariograms · Bryophytes

1 Introduction

The moss biomonitoring technique is widely used to characterise levels of atmospheric contamination. The technique was developed at the end of the 1960s as a study tool to

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investigate the atmospheric deposition of heavy metals (Rühling and Tyler 1968; Tyler and Rühling 1970). At present, the technique is most commonly used in the "Heavy Metals in European Mosses" project (Harmens et al. 2008) in which a large number of European countries participate. The protocol proposed by Rühling (1989) is used in the project, and only minimal modifications have been made to produce the current version (Harmens 2005). In the first stage of the protocol—collection of the moss samples—selection of the size of the sampling site (where a series of subsamples are collected) is a key factor, as this may determine the results obtained. In the above-mentioned protocol, the size of area recommended is 50×50 m (i.e. 2,500 m^2). However, we were unable to find any references in the relevant literature in which any justification is provided for the use of this size of site. In addition, there is no agreement in this respect, as other authors such as Cenci (1998) recommend different sampling site sizes (i.e. maximum area less than 50 m^2, which for compact moss mops, may be reduced to 1 m^2). Fernández et al. (2002) recommended another size of sampling area (i.e. approx. 35×35 m) because of the characteristics of the study zone (Galicia, NW Spain). This region has 2,217,140 ha of agricultural land comprising 5,445,081 plots of average area 0.4 ha, as well as a highly dispersed population (29,947 centres of population in 29,575 km^2) where it is difficult to find areas of the recommended dimensions that also fulfil the other criteria.

In addition to the recommendations regarding the size of the sampling sites, the protocol also include recommendations about the distance from tree cover where the moss samples should be collected. The fulfilment of both requisites may lead to new problems, as according to the protocol, when the samples are not collected from open areas, they should be collected from small clearings in forests, avoiding mosses that grow close to trees. Thus, for distance from the tree of 3–5 m, and a sampling site of 50×50 m, the area of a circular clearing would vary between 4,632 m^2 and 5,128 m^2. Another factor not taken into account and which would determine the area where mosses may be collected is the "shading effect" of any vegetation adjacent to the clearing. According to the recommendations of the World Meteorological Organization, when siting a pluviometer, for correct measurement of precipitation, the distance from any object in relation to itself should be more than twice its height (WMO-No.8, 1983). Following this reasoning, this "shading effect" should be taken into account when collecting moss samples for determination of the deposition of contaminants. For example, if the vegetation is 20 m in height, the sampling area should be at a distance of at least 40 m, and therefore the diameter of the forest clearing should be 40+71+40 m, i.e. a minimum surface area of 17,837 m^2. It is difficult to encounter forest clearings of the required dimensions, as in the first case, the area required would be the equivalent of approximately 0.5 ha, and in the second more restrictive case, it would be equivalent to 2 ha. However, in a meta-analysis carried out in Germany by statistical analysis and use of a geographic information system, it was concluded that there is a weak relationship between the bioconcentration in mosses and the distance from vegetation (Pesch and Schröder 2006), and therefore more studies are required in order to investigate the effect of this distance and tissue uptake of elements by moss.

Despite the effect that we assume that the selection of the size of sampling area has on the results obtained, no scientific studies have yet been carried out to clarify this aspect. Furthermore, in approximately half of the references on biomonitoring of heavy metals in native terrestrial mosses (some 270 such articles were located at www.scopus.com), the authors do not specify the size of the area in which the samples were collected (Fig. 1). However, the size of sampling site most commonly used (when specified) is 50×50 m, as recommended in the previously mentioned protocol.

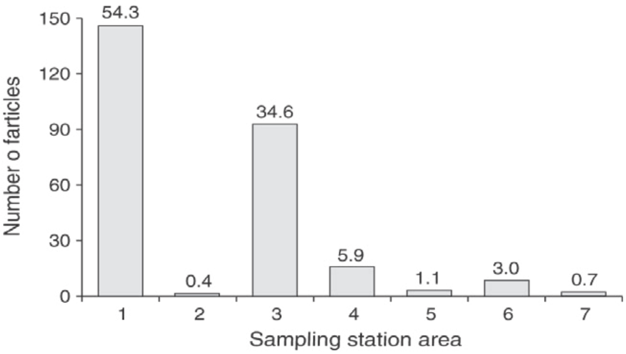

Fig. 1 Number of scientific articles on the biomonitoring of heavy metals with native terrestrial mosses, and the percentage (%) corresponding to each of the different classes established according to the length of the side of the sampling site (SS): 1=not specified; 2=100 m; 3=50 m; 4=50–20 m; 5=20–10 m; 6=10–2 m; 7=<2 m

Considering as the null hypothesis the existence of spatial independence between the concentrations of As, Cd, Cu, K, Hg, Ni, Pb, Se and Zn in the moss *Pseudoscleropodium purum* collected from several sampling sites, the objective of the present study was to propose a method of determining the optimal size of sampling site for the moss biomonitoring technique.

2 Material and methods

2.1 Sampling

Samples of the moss *Pseudoscleropodium purum* (Hedw.) M. Fleisch were collected from three sampling sites (SS); these sites are representative of different pollution levels and were selected following the results of previous studies. The aim of this selection was to test how these levels affect the spatial structure of moss concentrations and not to represent a large area. The first, SS1, of dimensions 30×30 m (coordinates: X=49,300, Y=475,834; UTM 29T ED50), is situated in the surroundings of an iron smelter and is contaminated by exceptionally high levels of As and Cu (Fernández et al. 2004, 2007). The second, SS2, of dimensions 25×20 m (X=60,706, Y=479,497; UTM 29T ED50), is located in the surroundings of a coal-fired electricity generating station, contaminated by moderate levels of As, Cd, Cu, Hg, Pb and Zn (Couto 2002; Fernández et al. 2002; Couto et al. 2003). The third site, SS3, of dimensions 25×30 m (X=58,640, Y=471,040; UTM 29T ED50), is located in a rural environment, distant from any industrial installations or population nuclei, and with low levels of contamination (Aboal et al. 2004). There is no tree cover in any of the SS, only grass and/or a few shrubs (i.e. *Ulex* spp., *Erica* spp. and *Pteridium aquilinum*).

A total of 50 samples of moss were collected in each SS. For this purpose, a regular 1× 1 m sampling grid was placed within the SS, with the nodes positioned at random. If there was no moss growing at any of the nodes, another was chosen and the procedure was continued until 50 samples were obtained, each consisting of the moss present within a radius of 25 cm from each node. All sampling was carried out between 15 May and 15 June 2003 to minimize any possible effects of temporal variation on comparing the different SS. A similar sampling survey had been carried out at SS2 in 2000, in which each sample consisted of isolated moss mops separated by at least 0.45 m; the results of this study were also used in the present study (SS4).

2.2 Chemical analysis

Prior to analysis of the moss, the samples were cleaned and apical segments (of length 3–4 cm) were cut from the shoots. The segments were then washed in bidistilled water for 30 s to remove remains of adhered material and deposited particles. The washed segments were then dried in an oven at 45°C and homogenized in an ultracentrifugal mill (Retsch ZM100). Finally the samples were digested in nitric acid (65% HNO_3, analytical grade), in a microwave oven (CEM MDS 2100).

The concentrations of Copper (Cu) and Zinc (Zn) were determined by flame atomic absorption spectrometry (Perkin Elmer 2100) and that of Potassium (K) by atomic emission spectrometry. Cadmium (Cd), Nickel (Ni) and Lead (Pb) were determined by graphite chamber atomic absorption spectrometry (Perkin Elmer AAnalyst 600), and Arsenic (As), Mercury (Hg) and Selenium (Se) by atomic fluorescence spectrometry (PSA Excalibur). The concentrations of As, Cd, K and Se were not determined at SS4 in 2000 because there were not enough sample for analysis of these elements.

To control the analytical quality of the extraction process and determination of the metals, certified reference material (GBW07604 poplar leaves; Institute of Geophysical and Geochemical Exploration, Langfang, China) and an internal reference material (O.1. *Pseudoscleropodium purum*), were included (one or both) every nine samples. The percentage recovery of the reference material was satisfactory in all cases (80–100%). The results of the analysis of the internal reference material were used to calculate the variability associated with the extraction and determination processes. This variability, expressed as the coefficient of variation, was 4% for K, 6% for Cu, Pb and Zn, 7% for Hg and Ni, 8% for As and Cd, and 10% for Se. Analytical blanks were also included, one every nine samples, to control for possible contamination.

2.3 Statistical analysis

To study the spatial structure in the concentrations of the elements in each SS, semivariograms were constructed by use of a robust estimator to calculate the semivariance (Cressie and Hawkins 1980). The following criteria were used to select the number and interval of the lags: (i) for each lag, the largest possible number of pairs of points was considered, maintaining the distribution of the pairs of points between lags as equal as possible (Webster and Oliver 1992; Bringmark and Bringmark 1998; García 1999); (ii) an attempt was made to consider at least 50 pairs of points for each lag (García 1999); (iii) an attempt was made to consider at least 10 lags (García 1999), and (iv) the lower limit for the first class was the zero distance, and upper limit of the last class was equal to half the distance of the longest dimension of the study area; the lag distance was the same for the intermediate classes. In the present study, we chose to use 10 lags with an interval of 2.25 m, varying the number of pairs of points per lag between 50 and 216, with the exception of two lags (with 30 and 49 pairs of points).

In order to confirm the existence of spatial structure, a method of randomizing the positions of the SS (10,000 times) was used and the distribution of the semivariances of the semivariograms was obtained under the hypothesis that there was no spatial structure. Comparison of the semivariances obtained with the data for this distribution enabled assignation, to each value, of the probability of appearance when there is no spatial structure. In accordance with this, all lags below and above the 2.5 and 97.5% quantiles respectively were considered significant. The 95% confidence intervals were calculated for the distribution of the 10,000 semivariances obtained for each lag. A computer programme (Delphi 3, Borland International 1997, modified by Aboal et al.

(2006b)) was used to calculate standard and robust variograms and to generate of the randomized distributions.

Finally, the semivariograms of the elements with several significant semivariances were modelled (SPSS, version 17.0) to find out which model (linear, exponential, spherical, etc.) provided the best fit on the basis of the coefficient of determination (Isaaks and Srivastava 1989).

In order to determine the percentage of the nugget effect that was due to the analytical error for each element, the semivariance of the concentrations of the analytical duplicates was calculated, assuming a distance of 0 m between pairs.

3 Results

The descriptive statistics of the elements determined in the moss collected at the SS studied are shown in Table 1. The median values of the concentrations of As, Cu, Hg, Ni, Pb and Se in samples from SS1—situated in the surroundings of an iron smelter—were between 2 and 10 times higher than in those from SS2 and SS3, depending on the element considered. For Cd, the median concentrations in samples from SS1 and SS3 were higher than in those from SS2. Finally, the median concentrations of K and Zn were similar in all three SS. Comparison of data from SS2 and SS4, which correspond to the same site sampled 3 years apart, revealed that in 2000, the median concentrations of Cu, Ni and Zn were at least two times higher than in 2003, whereas the concentrations of Hg and Pb were similar in both sets of samples.

The robust semivariograms corresponding to SS1 (Fig. 2) include the mean semivariance, the region of acceptance of the hypothesis of absence of any spatial structure in the data (established from the 2.5 and 97.5% quantiles), and when there was spatial

Table 1 Descriptive statistics of the concentrations (μg g^{-1}; ^{a}: ng g^{-1}) of different elements in samples of the moss *Pseudoscleropodium purum* collected from four sampling sites (SS) in Galicia (NW Spain). Mad.: median absolute deviation; Min: minimum value; Max.: maximum value; -: not determined

		As^{a}	Cd^{a}	Cu	Hg^{a}	K	Ni	Pb	Se^{a}	Zn
SS1	Median	166	116	22.1	33.6	4,348	6.82	3.91	286	45.7
	Mad.	45	16.2	2.43	6.65	704	1.10	1.07	43	5.80
	Min	34.0	64.4	16.4	12.2	2,618	3.70	0.940	170	32.7
	Max	397	170	43.8	86.8	6,357	13.8	6.26	409	60.4
SS2	Median	50.8	51.2	3.92	28.9	6,880	0.955	1.34	99.0	37.4
	Mad.	14.5	12.2	0.590	13.1	1,089	0.345	0.44	12	4.40
	Min	21.4	28.6	2.50	12.0	4,513	0.400	0.360	52.0	24.2
	Max	80.8	74.5	5.30	45.9	9,248	1.52	2.35	146	50.6
SS3	Median	43.3	83.4	3.02	16.9	4,888	0.665	1.72	90.5	46.0
	Mad.	7.30	17.6	0.255	2.05	501	0.125	0.250	17.5	5.20
	Min	22.9	49.2	2.50	10.6	3,731	0.440	0.800	44.0	28.1
	Max	94.7	236	4.95	29.6	6,739	2.20	3.25	160	64.2
SS4	Median	–	–	10.2	22.6	–	1.43	1.27	–	65.6
	Mad.	–	–	2.85	3.65	–	0.550	0.355	–	12.3
	Min	–	–	5.40	9.08	–	0.190	0.110	–	35.4
	Max	–	–	36.7	47.4	–	3.65	8.90	–	152

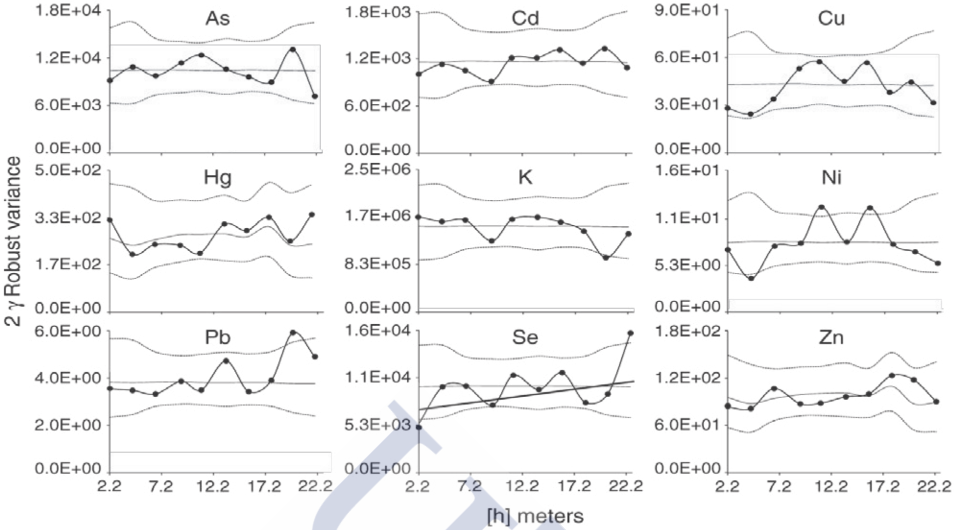

Fig. 2 Semivariograms of the concentrations of several elements in the terrestrial moss *Pseudoscleropodium purum* in sampling site 1. The continuous line represents the mean semivariance, the dashed lines represent the 97.5% and 2.5% quantiles, and the thick continuous line represents the fitted model in those cases in which there was spatial structure in the data

structure, the corresponding model fit. The first lag was found to be outside the region of acceptance only for Se, demonstrating spatial structure in the corresponding data, which fitted a linear model.

The robust semivariograms for SS2, SS3 and SS4 are shown respectively in Figs. 3, 4 and 5. Spatial structure was only observed for Cu and Zn in SS2, and Hg and K in SS3, and the data fitted a linear model. There was spatial structure in the data for Hg and Pb in SS4, which fitted a spherical model. For the other elements, in all SS and on all dates, all of the lags were within the region of acceptance of the null hypothesis and there was no spatial structure in the data.

The parameters corresponding to the model fits for those elements that displayed spatial structure, are shown in Table 2. C_0 is the nugget, or value of the semivariance when the distance is zero. In the spherical models, C_1 is the sill or the total semivariance of the semivariogram and a_0 is the semivariogram range or autocorrelation range. In the linear models, b represents the slope of the straight line. The values of the regression coefficients of these models are generally higher than 0.60, except for Se in SS1 and Hg in SS4 (0.25 and 0.58, respectively).

The percentage nugget effect due to analytical error is shown in Table 3. For As, Cu, Hg and Ni, the error was different in the three SS (SS3 > SS2 > SS1), and varied between 5.6 and 62.7%. For Cd, Pb, Se and Zn, the highest error values corresponded to SS2, and for K, to SS3.

4 Discussion

The moss biomonitoring technique was originally developed in Scandinavian countries and the recommendations in the protocol may be well suited to this region, where there are large

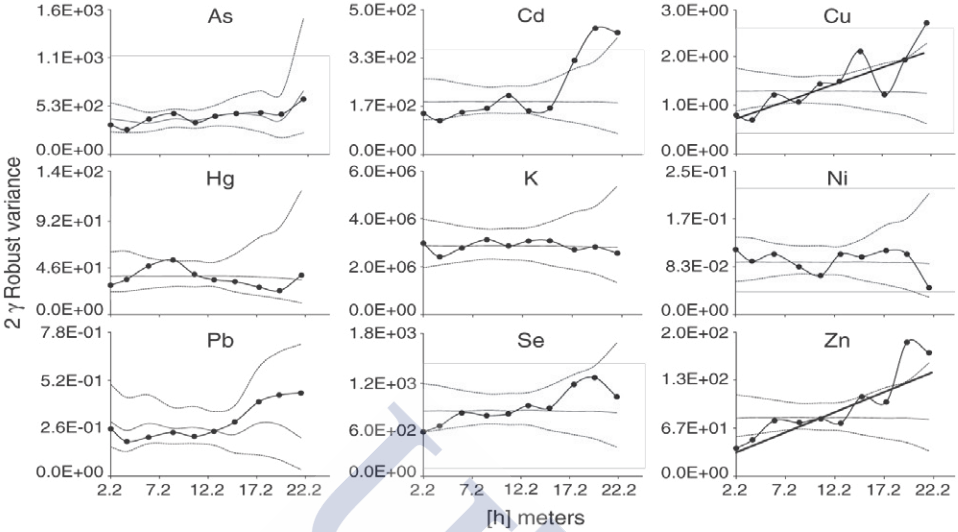

Fig. 3 Semivariograms of the concentrations of several elements in the terrestrial moss *Pseudoscleropodium purum* in sampling site 2. The continuous line represents the mean semivariance, the dashed lines represent the 97.5% and 2.5% quantiles, and the thick continuous line represents the fitted model in those cases in which there was spatial structure in the data

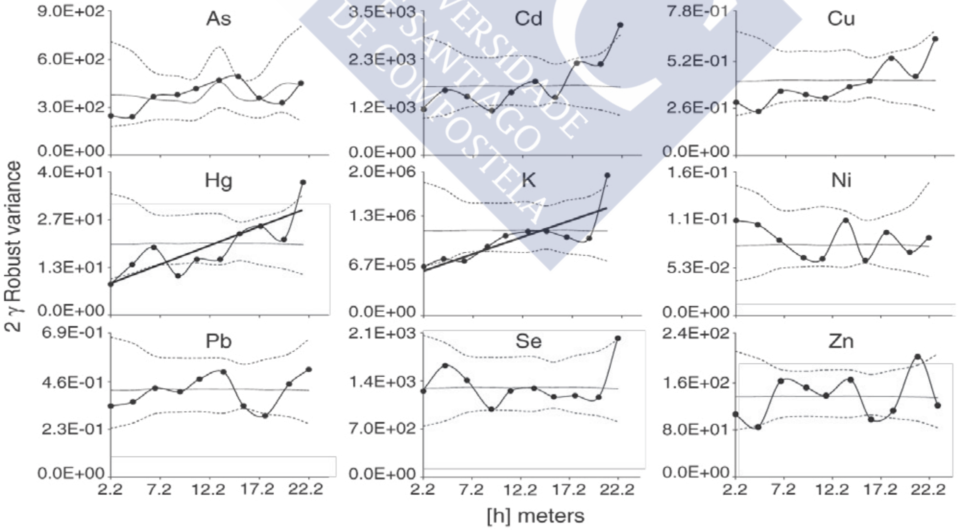

Fig. 4 Semivariograms of the concentrations of several elements in the terrestrial moss *Pseudoscleropodium purum* in sampling site 3. The continuous line represents the mean semivariance, the dashed lines represent the 97.5% and 2.5% quantiles, and the thick continuous line represents the fitted model in those cases in which there was spatial structure in the data

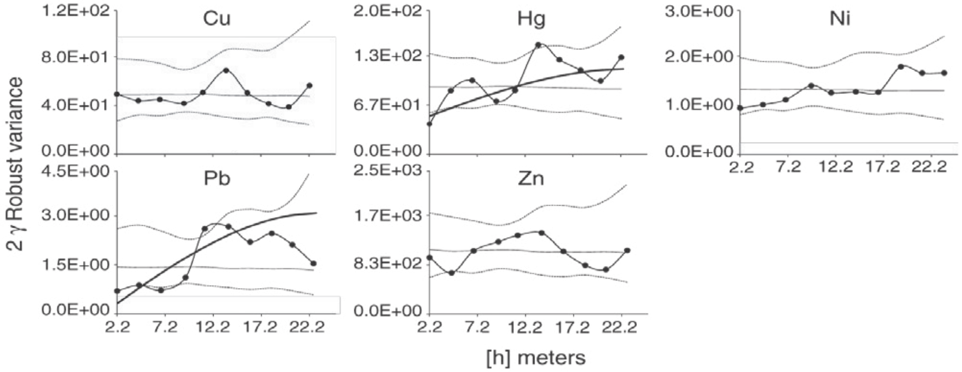

Fig. 5 Semivariograms of the concentrations of several elements in the terrestrial moss *Pseudoscleropodium purum* in sampling site 4. The continuous line represents the mean semivariance, the dashed lines represent the 97.5% and 2.5% quantiles, and the thick continuous line represents the fitted model in those cases in which there was spatial structure in the data

open spaces covered by herbaceous vegetation or shrubs. However, such areas are not found in other more southerly regions, where the technique was later applied. In the latter regions, sampling sites of the recommended size may be difficult to encounter. This problem is aggravated by the need to comply with the recommendations as regards to the distance from any tree cover that samples can be collected. We reviewed the relevant literature and found that little importance was attached to the size of the sampling area, as in many studies it is not even specified, and in most of the others a sampling area of 50 × 50 m is used, without any justification for selection of this size of sampling area.

One way of solving the problem of determining the dimensions of the sampling station is to study the spatial variation in the concentrations of elements in moss, at the sampling site level, by the individual analysis of samples collected in the SS, which usually correspond to subsamples used to make composite samples. Spatial autocorrelation may occur at different spatial scales: local, regional, hemispheric and global, as demonstrated by Holy et al. (2010) and Schröder et al. (2010a, b).

Table 2 Semivariogram parameters fitted to the data for the elements measured in samples of the moss *Pseudoscleropodium purum* collected from four sampling sites (SS) in Galicia (NW Spain) and that display spatial structure. C_0: semivariance at zero distance or nugget; C_1: total semivariance of the semivariogram; a_0: range in meters; b: slope of the straight line; r^2: regression coefficient

	Element	Model	C_0	C_1	a_0	b	r^2
SS1	Se	Linear	6,327	–	–	222.1	0.25
SS2	Cu	Linear	0.485	–	–	0.079	0.69
	Zn	Linear	15.7	–	–	6.49	0.79
SS3	Hg	Linear	6.62	–	–	0.983	0.64
	K	Linear	527,221	–	–	41,993	0.62
SS4	Hg	Spherical	40.0	82.7	18.5	–	0.58
	Pb	Spherical	−0.240	2.61	16.0	–	0.75

Table 3 Percentage of the nugget effect due to analytical error in the elements measured in the samples of the moss *Pseudoscleropodium purum* collected at four sampling sites (SS) in Galicia (NW Spain)

Element	Contribution of the analytical errors in the nugget value (%)			
	SS1	SS2	SS3	SS4
As	2.40	59.07	65.13	–
Cd	1.95	11.54	1.29	–
Cu	0.14	7.66	14.38	0.13
Hg	1.15	7.97	34.57	7.24
K	34.72	17.20	74.79	–
Ni	0.06	4.92	5.68	0.34
Pb	3.23	42.26	26.91	16.19
Se	7.33	47.35	29.17	–
Zn	28.69	72.97	20.45	2.67

Thus, in the present study, the results obtained revealed three different possibilities: (i) the existence of spatial independence in the concentration of the elements determined; (ii) the existence of spatial structure, with a linear model fit, and (iii) the existence of spatial structure, with a spherical model fit.

In 25 of the 32 cases studied, there was no spatial structure in the data (Figs. 2, 3, 4 and 5), in other words, there was no defined pattern of variation in the concentration in relation to distance and therefore the size of the sampling area cannot be delimited. In such cases, the entire semivariogram is subject to the nugget effect, which may be due to: i) errors associated with sampling and analytical determination; or ii) the existence of spatial dependence that occurs at a smaller scale than the distance from the first lag (i.e. 2.25 m). In regard of the sampling error, as all of the moss within a circular area of radius 25 cm from the node was collected, no error was introduced. The analytical error was generally very low (Table 3), and was lower for those elements present at high concentrations than those present at low concentrations (Table 1). Thus, when there was a high degree of contamination, there was a high degree of variation in the concentration between samples, and therefore in relative terms the variation associated with analytical error was minimal. By contrast, when there was almost no contamination, the degree of variation between samples was low and the analytical error was proportionally higher. Thus, in SS1, the percentage error was low for the elements with a high degree of contamination, such as: As, Cd, Cu, Hg, Ni, Pb and Se, and in contrast, the percentage error was high for K and Zn, which were present at low concentrations. The same was true for SS2. However, as the level of contamination in SS3 was low (Table 1), the values of the associated error were higher. These findings lead us to assume that the most likely cause of the absence of spatial structure in the range of distances studied is that the structure exists at a shorter distance than the first lag. Thus when no spatial structure is detected, the concentrations of elements in the subsamples (samples in the present study) would be spatially independent, and therefore the distance between them, at least for the range of distances studied, will not determine the final result obtained, so that the dimensions of the sampling site (when less than 43 m) would not affect the concentrations in the composite sample.

There was spatial structure in the data for Se in SS1 (Fig. 2), Cu and Zn in SS2 (Fig. 3), and Hg and K in SS3 (Fig. 4), and the data fitted a linear model. The dimensions of the sampling site, within the range of distances studied are also irrelevant in this case and the results did not vary, independently of whether the dimensions were e.g. 20×20 or 50×50 m. There are different possible reasons for a linear model fit for the pattern of variation between samples: i) if we assume that the pattern fits an inclined plane (i.e. drift), the concentration in the composite sample will correspond to the mean value for the subsamples (treated as samples in the present study) and will coincide with the mid point of the sampling site, irrespective of its dimensions; and ii) the spatial scale of the process may be greater than the sampling area, and thus at greater distances than those studied, the linearity will be lost. However, in the study area as such distances were not reached, the linearity was maintained.

Finally, there was spatial structure in the data for Hg and Pb in SS4, which fitted a spherical model (Fig. 5); this enabled the size of the sampling area to be defined. The definition was made on the basis of the range of autocorrelation of these elements, by selecting the most restrictive value of the range (a_0), which in this case was 16 m. Above this value, the samples would be spatially independent and therefore any increase in distance would not affect the results. Therefore, regarding the three possible options, if the diameter of the sampling site was greater than 16 m, this would be sufficient, and therefore large sizes such as between 20×20 and 50×50 m would be valid.

In regard to the form of the sampling site, on the basis of the results obtained, which usually display spatial independence, there is no reason why these must be square. The best option is perhaps a circular area of diameter greater than 16 m (i.e. minimum area, 201 m^2), as it is fairly easy to find open spaces of these dimensions. The centre of the sampling site can be recorded with a GPS, and the moss samples collected from within the selected area.

In light of the results obtained for the sites and species used, and after finding that in most cases there was no spatial structure, or that it was linear, we can conclude that selection of the size of the sampling site does not determine the results obtained when subsamples are combined to make a composite sample. Spatial structure was occasionally detected, with a corresponding spherical model fit, therefore determining that the size of the sampling sites must be more than 16 m in diameter. The maximum limit can be established as the diameter corresponding to the 50×50 m sampling site (i.e. 71 m). However, within these dimensions, other factors such as the number of subsamples collected and the equilibrated weight of the samples, and not the size, will determine the final result (Fernández et al. 2002; Aboal et al. 2006a). Finally, although the suggestion in the original protocol of collecting composite samples over an area of 2,500 m^2 is sensible, it may be overcautious as an area of 200–250 m^2 appears to be sufficient.

5 Conclusions

For those elements that do not display any spatial structure, or linear spatial structure, i.e. almost all of the cases considered, the size of the sampling area did not affect the results obtained, and the dimensions of the area were irrelevant within the range studied. Thus, other factors such as the number of subsamples collected or the equivalent weights (as detailed in the relevant literature), would have determined the results obtained.

The spherical model fit for the existing spatial structure enabled definition of the range of autocorrelation, which in turn enabled the size of the sampling area to be determined, which in the present study corresponds to an area of diameter >16 m. The proposed method must be applied to other species and other regions to determine the size of sampling area that should be used in the moss biomonitoring technique.

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CHAPTER 02


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On the Use of Epigaeic Mosses to Biomonitor Atmospheric Deposition of Nitrogen

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Abstract In this study, we investigated whether the terrestrial moss *Pseudoscleropodium purum* can be used to biomonitor atmospheric deposition of nitrogen (N). For this purpose, we first determined whether there are any interspecific differences in the concentrations of total N and δ^{15}N between the two species of terrestrial moss most commonly used in biomonitoring studies, *P. purum* and *Hypnum cupressiforme*. Second, we determined the spatial distribution of N and δ^{15}N at small and large scales: (1) by analysis of 165 samples from the surroundings of an aluminium smelter and (2) by analysis of 149 samples from sites forming part of a regular 15 × 15-km sampling network in Galicia (northwest Spain). We did not find any interspecific differences in either total N or δ^{15}N. Analysis of δ^{15}N enabled us to identify large-scale spatial patterns of distribution that were congruent with the location of the main N emission sources (unlike the analysis of total N). However, we did not identify any such patterns for the small-scale source of N emission studied. The results show that analysis of δ^{15}N has an advantage compared with the analysis of total N in that it provides information about the source of N rather than about the amount of N received. Furthermore, isotope discrimination appears to occur, with the bryophytes preferentially accumulating the N^{14} isotope. Although this amplifies the signal of reduced forms, it is not problematical for determining spatial-distribution patterns.

Terrestrial mosses have been used to biomonitor heavy-metal contamination since the end of the 1960s (Rühling and Tyler 1968; Tyler and Rühling 1970). Terrestrial mosses have also been tested for their potential usefulness in biomonitoring other contaminants, various types of organic compounds, such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls, polychlorinated dibenzo-p-dioxins, and polychlorinated dibenzofurans (e.g., Knulst et al. 1995; Lead et al. 1996; Orlinski 2002; Gerdol and Bragazza 2006; Carballeira et al. 2006), metal isotopes (e.g., Kunert et al. 1999), radioactive elements (e.g., Papastefanou et al. 1989, 1992; Triulzi et al. 1996; Sawidis et al. 1999, 2009; Tsikritzis et al. 2003), halogens, such as fluorine (F) (e.g., Real et al. 2003), heavy metals (Fernández and Carballeira 2000; Tsikritzis et al. 2002; Shakya et al. 2007, 2008), and nonmetals, such as N (e.g., Harmens et al. 2011).

During the last few decades, there has been an increase in emissions of N to the atmosphere as a result of the worldwide increase in demand for foodstuffs and energy. Thus, the atmospheric deposition of N originating from anthropogenic activities has become an important and predominant source of N in some ecosystems (Erisman et al. 1998; Galloway et al. 2008). In fact, the global depositions of anthropogenic N now equal those from natural sources, giving rise to a two-fold increase in the amount of N available to organisms in less than a century (Vitousek et al. 1997). The first research relating the atmospheric deposition of N to the body burden of this element in terrestrial mosses was performed by Pitcairn et al. (1995) in the United Kingdom. Other investigators have continued to work on this topic in field studies (e.g., Pitcairn et al. 2006; Poikolainen et al. 2009; Schröder and Pesch 2010; Harmens et al. 2011; Stevens et al. 2011a, b) and in laboratory-based studies (e.g., Leith et al. 2005;

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Solga and Frahm 2006; Salemaa et al. 2008; Stevens et al. 2011b).

Although the studies performed to date have shown only a weak relationship between N deposition and accumulation in moss tissues (e.g., Pitcairn et al. 2006; Solga et al. 2005; Stevens et al. 2011a), it is possible to use mosses to identify areas exposed to N contamination (Harmens et al. 2011). One possible explanation for the weak relationship between the deposition and accumulation of N is the regulation of tissue loads in mosses because N is widely known to play an important role in bryophyte metabolism (e.g., Koranda et al. 2007; Arróniz-Crespo et al. 2008). Such regulation may distort the patterns of N deposition identified by biomonitoring with terrestrial mosses. In addition, the degree of regulation may depend on the species of moss, which would generate different rates of accumulation of N deposition (Salemaa et al. 2008; Arróniz-Crespo et al. 2008). Thus, Schröder and Pesch (2010) indicated that the use of different species of moss in extensive surveys may distort the spatial patterns.

Determination of the contents of the stable isotopes of N (^{14}N and ^{15}N) in moss tissues has recently been suggested as an alternative to determining the total concentration of N in tissues (Pearson et al. 2000; Solga et al. 2005; Liu et al. 2008a, b, c; Zechmeister et al. 2008). Both isotopes are found globally in a constant proportion in the atmosphere (99.63 % of ^{14}N and 0.36 % of ^{15}N [Nier 1950]). However, it has been shown that the ^{15}N/^{14}N ratios (δ^{15}N) are different in oxidised (NO_x) and reduced (NH_y) forms of N and that they are higher in the former (Freyer 1978; Garten 1992; Heaton et al. 1997). Analysis of δ^{15}N enables the identification of diverse sources of N emission of anthropogenic origin. The oxidised forms of N, such as N_2O and NO_x, are mainly emitted to the atmosphere by transport, industry, and energy production, with the latter contributing 70 % of the emissions of oxidised compounds (Bragazza et al. 2005); the reduced forms mostly originate from cattle-breeding and the use of fertilizers in agriculture. The most positive δ^{15}N signatures have been recorded in samples of moss from urban areas and the most negative in samples of moss from rural areas (Pearson et al. 2000; Solga et al. 2005; Liu et al. 2008a, b; Zechmeister et al. 2008). Thus, if δ^{15}N signatures are affected by isotope discrimination in N uptake, the spatial patterns of deposition (which would not enable quantification of the levels of N deposition) would not be distorted by regulatory mechanisms and thus would provide information about the sources of emission of N.

The following hypotheses were tested in the present study: (1) there are no interspecific differences in the concentrations of either total N or δ^{15}N between the two species of terrestrial moss usually used in monitoring surveys; and (2) there is no spatial structure in either the total concentrations of N or the concentrations of δ^{15}N in the surroundings of large- or small-scale sources of N emission.

Material and Methods

Sampling and Processing

The species used in the present study were *Pseudoscleropodium purum* (Hedw.) M. Fleisch and *Hypnum cupressiforme* Hedw. Whenever possible, samples of the mosses were collected at a distance of at least 300 m from main roads and centres of population and at least 100 m from other types of roads and isolated houses. The samples were collected in open areas or in small clearings in forests, and sampling close to trees was avoided. Approximately 30 subsamples of similar weight were collected at each sampling site (SS) and combined to form a single composite sample (Fernández et al. 2002; Aboal et al. 2006a). Whenever possible, the size of the sampling area was between 30 × 30 and 50 × 50 m.

The apical sections (length 3 to 4 cm) of all samples were separated from the basal portions and maintained in a moisture-saturated environment (10 °C) for 1 week. The apical sections were then washed with shaking (30 s) in double-distilled water to remove other adhered plant material. The washed apical sections were then dried in an oven at 45 °C. Finally, the material was homogenized in an ultracentrifuge mill (Retsch ZM100, Retsch GmbH, www.retsch.com) and stored at room temperature in the dark until analysis.

Interspecies Comparison

For comparison of the N with the δ^{15}N contents in the two species of moss chosen for study, 20 samples of *H. cupressiforme* and 20 samples of *P. purum* were collected from the same SSs located close to industrial sources of emission and in areas with low levels of contamination in Galicia (northwest Spain). The samples were collected between 1997 and 2007.

Regional-Scale Spatial Structure

To determine the patterns of distribution of N and δ^{15}N, samples of the terrestrial moss *P. purum* were collected in March 2004 from each of the nodes of a regular 15 × 15-km sampling network formed by 149 SSs in Galicia (northwest Spain) (Fig. 1a). More than 70 % of the population of Galicia lives on the Atlantic coast (population density data are included in Fig. 1b, c). There are also three coal-powered electricity-generating plants in the region (Fig. 1b, c), which according to the European Pollutant Release and Transfer Register (E-PRTR Spain) emitted >20,000 ton year^{-1} of NO_x/NO_2 to the atmosphere in 2004. Of these emissions, 95 % originated from the As Pontes power plant, situated in the north of the study area. Furthermore, there are another three electricity power plants in adjacent regions (Fig. 1b, c), which together emit a total of 64,400 ton of NO_x/NO_2/y, of which 56 % is attributed to the Compostilla power plant located in eastern Castilla-León. Moreover, the area also includes 2,041,800 hectares of agricultural land under different types of use and approximately 70,000 livestock farms, including >970,000 cows and 1,150,000 pigs (Instituto Galego de Estatística [http://www.ige.eu/]) in 2009. A high input of reduced N compounds in the ecosystems is therefore expected.

To facilitate interpretation of the results obtained, we have included maps of the regional-deposition models for reduced and oxidised N (Fig. 1b, c, respectively). These models were elaborated by the European Monitoring and Evaluation Programme (EMEP) with the EMEP emission data for Europe (which also include industrial data from the previously cited E-PRTR inventory) and the meteorological data obtained by the European Centre for Medium-Range Weather Forecasts. The model does not include immision or deposition measures, and therefore the results obtained may be of limited robustness in relation to the present study.

Small-Scale Spatial Structure

In 2004, 165 samples of *P. purum* were collected from the surroundings of an aluminium smelter, Alumina Española, located on the northern coast of Galicia (Fig. 1b, c). Taking the focal point of N emission in the smelter as the starting point, a total of 8 sectors were established around the smelter, with the exception of the northern area, which is bound by the sea. The angle of each sector was 30°, and the maximum distance from the focal point of emission was 5,000 m. Between 6 and 32 SS were located within each sector (Fig. 1d). According to E-PRTR Spain, the factory emitted 1,770 ton of NO_x/NO_2 in 2004. Two of the sectors (nos. 3 and 5) were sampled again in 2007, a year in which the factory emitted 2,830 ton of NO_x/NO_2.

Chemical Analysis

Aliquots (approximately 3 mg) of the samples were weighed out and packed into tin capsules (EuroVector). The capsules were stored in a desiccator until N and $\delta^{15}N$ analysis (performed in the Unidad de Tecnicas Instrumentales de Analisis, Servicios de Apoyo a la Investigacion, University of A Coruña). The samples were combusted in an elemental analyser (FlashEA1112; ThermoFinnigan) coupled to an isotopic ratio mass spectrometer (Deltaplus; ThermoFinnigan). The reference standard used for quantifying the N content was acetanilide. Calibration of the reference gas (N_2) for atmospheric ^{15}N was performed with IAEA-N-1 ($(NH_4)2SO_4$), IAEA-N-2 ($(NH_4)2SO_4$), and IAEA-NO-3 (KNO_3) as standards. The isotope ratios ($^{15}N/^{14}N$) in the samples were expressed relative to the standard ratio (atmospheric N_2) so that the proportions obtained were comparable. The relative abundance of ^{15}N in the sample ($\delta^{15}N$) was calculated from the following formula (Eq. 1):

$$\delta^{15}N\ (‰) = \left[\left(R_{sample}/R_{standard}\right) - 1\right] \times 10^3 \qquad (1)$$

where R is the $^{15}N/^{14}N$ ratio. The global error, determined by the use of analytical replicates ($n = 9$) was 8 %.

Statistical Analysis

The differences between N and $\delta^{15}N$ for *H. cupressiforme* and *P. purum* were studied by type II standard major axis regression. A one-sample test of a (standardised) major axis slope was also performed to determine whether the slopes of the regression lines were significantly different from 1 (Zar 1984). The same test was also used to determine whether the elevations of the regression lines were significantly different from 0. All analyses were performed with R software(version 2.11.0).

The density functions for N and $\delta^{15}N$ on a regional scale were determined by kernel smoothing using the KernSmooth software package (Ripley 2002) and R software (R Development Core Team 2008). Kolmogorov-Smirnov test was applied using the same statistical package to test the normality of the distributions.

To detect any spatial structure in N and $\delta^{15}N$ data, semivariograms were constructed by use of a robust estimator of the semivariance (Cressie and Hawkins 1980). According to Aboal et al. (2006b), a randomization method was used to determine whether the semivariograms were significant. When spatial structure was detected, the large-scale trends were estimated by median polish (Cressie 1986). The suitability of this technique for analysis of the data obtained in this type of sampling network has been shown for terrestrial mosses (Aboal et al. 2006b; Boquete et al. 2009). To test for the existence of a large-scale random component, the data were analysed (as described previously) to determine whether the median polish residuals displayed any spatial structure. Finally, linear interpolation of the values estimated by median polish was performed to map the study zone using ArcGIS 9.2 software. All data sets were analysed to identify any significant correlations between the total concentrations of N and $\delta^{15}N$ using R software (R Development Core Team 2008).

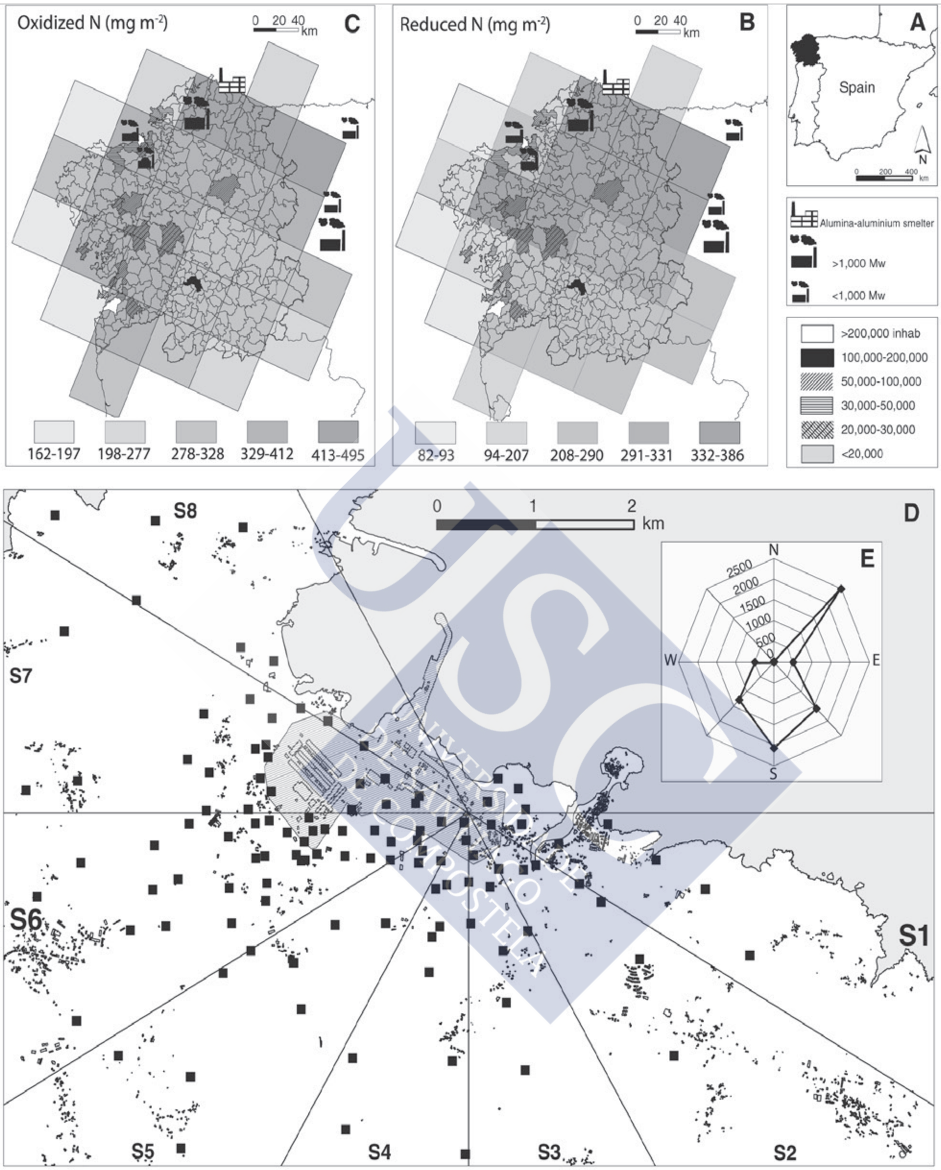


Fig. 1 **a** Location of Galicia (northwest Spain). **b** Values of reduced N (mg m^{-2}) obtained from regional-deposition models, location of coal-fired power plants in the region and surroundings, location of the alumina–aluminium smelter under study, and the distribution of the population in the region. **c** Values of oxidised N (mg m^{-2}) obtained from regional-deposition models, location of coal-fired power plants in the region and surroundings, location of the alumina–aluminium smelter under study, and the distribution of the population in the region. **d** Location of sampling stations around the alumina–aluminium smelter and the assignment of sectors (SS nos. 1 to S8). **e** Wind rose for the study area, constructed from annual hourly wind data. The thick line represents hours of wind

Results

There were no significant correlations between the total concentrations of N and $\delta^{15}N$ in any of the data sets.

Interspecies Comparison

The variability in $\delta^{15}N$ in *P. purum* and *H. cupressiforme* (coefficients of variation of 73 and 68 %, respectively) was greater than the variability in N (coefficients of variation of 29 and 30 %, respectively). There were no significant differences between the concentrations of N in the two species of moss or between the $\delta^{15}N$ values (Fig. 3) because the slopes of the regression lines were not significantly different from 1, and the elevations were not significantly different from 0. However, the linear fits were not satisfactory given the low values of the coefficients of determination in both lines, particularly in the case of N ($r^2 = 0.24$*).

Regional-Scale Spatial Structure

The descriptive statistics of the results obtained for N and $\delta^{15}N$ on a regional scale are listed in Table 1 along with results obtained in previous studies. In Galicia, total N varied between 0.60 and 2.40 %, whereas $\delta^{15}N$ varied between −7.60 and −1.00 ‰.

The density function, the robust semivariogram, and the map of the spatial distributions of N and $\delta^{15}N$ in the different SS are shown in Fig. 2. The density function for N (Fig. 2a) does not follow a normal distribution and is skewed to the right because of the presence of a single outlier. The semivariogram of N concentrations does not display spatial structure, and the whole semivariogram is subjected to the nugget effect; the map of the distribution of concentrations does not show the existence of a spatial pattern. In contrast, the density function of $\delta^{15}N$ values (Fig. 1b) fits a normal distribution. The semivariogram displays weak spatial structure, and the first lag is outside the region of acceptance. In this case, the map corresponding to $\delta^{15}N$ (Fig. 2b) shows that the maximum values occurred in the northwest and southeast sectors of the region. The linear interpolation of $\delta^{15}N$ values estimated by median polish is shown in Fig. 2C; the highest values (*i.e.*, >−4.5 ‰) correspond to the densely populated Atlantic coast and to areas affected by electricity-generating stations in the northern and southeast sectors of the region. The results for the median polish include the already mentioned spatial structure of the $\delta^{15}N$ because the residuals do not display any spatial structure (Fig. 2c). The percentage of variance in the initial data explained by the median polish predictions was 21 %, which was calculated as the coefficient of determination of the regression of the raw data compared with the values predicted by median polish.

Small-Scale Spatial Structure

The values of N and $\delta^{15}N$ were plotted against the distance from the aluminium smelter for each of the sectors sampled (Fig. 4). The concentrations of N in the moss samples did not decrease in relation to the distance from the emission source during the period of the study. In sector 6, which was the only sector sampled in 2007, there was no decrease in the $\delta^{15}N$ values with distance from the emission source.

Table 1 Maximum, minimum, and mean concentrations of N (%) and $\delta^{15}N$ (‰) for different species in the present study and in other studies in Europe and China

Investigator	Species	Nitrogen or $\delta^{15}N$	*n*	Maximum	Minimum	Mean
Present study	*P. purum* (100 %)	Nitrogen	148	2.40	0.60	1.00
		$\delta^{15}N$	148	−1.00	−7.60	−4.70
Liu et al. (2008a, b, c)	*Haplocadium microphyllum* (−)	Nitrogen	175	2.24	1.27	–
	H. angustifolium (−)	$\delta^{15}N$	175	−1.39	−12.5	–
	Brachythecium salebrosum (−)					
	Eurohypnum leptothallum (−)					
Zechmeister *et al*. (2008)	*H. splendes* (50 %)	Nitrogen	490	1.99	0.76	–
	P. schreberi (30 %)	$\delta^{15}N$	490	−2.45	−10.04	–
	Abietinella abietina (10 %)					
	H. cuppresiforme (6 %)					
	P. purum (4 %)					
Solga *et al*. (2005)	*P. purum* (100 %)	Nitrogen	8	1.94	0.71	–
		$\delta^{15}N$	8	−2.98	−7.89	–
	P. schreberi (100 %)	Nitrogen	8	2.31	0.84	–
		$\delta^{15}N$	8	−2.86	−7.54	–

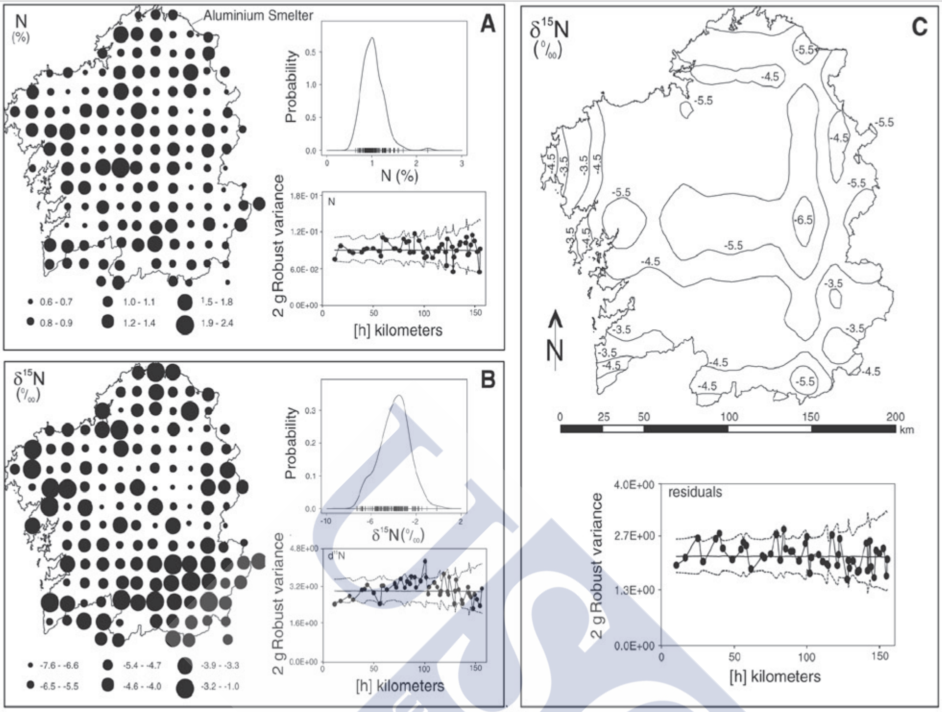


Fig. 2 **a**, **b** Concentrations, density functions, and semivariograms of N (%) and $\delta^{15}N$ (‰), respectively, for *P. purum* at a regional level. In each semivariogram, the *continuous line* represents the mean semivariance, and the *dashed lines* represent the 97.5 and 2.5 % semivariances, respectively. **c** linear interpolation (estimated by median polish) of $\delta^{15}N$ values with the corresponding semivariogram

Discussion

The total concentrations of N and $\delta^{15}N$ in the bryophytes studied provided different types of information because both variables varied independently in all of the data sets used. We must therefore establish which of the two variables used in moss biomonitoring would be the best for identifying patterns of atmospheric deposition of N.

The results obtained show that there are no interspecific differences in the concentrations of total N between the two species of moss under study. However, interspecific differences in heavy-metal concentrations, in the same species in the same study area, have been reported (Carballeira et al. 2008). The lack of interspecific differences in N suggests that there are no differences in the regulation of the levels of N in these species and that the rates of N accumulation are the same in both. Thus, the use of either or both of these species in extensive surveys would not distort the spatial patterns of N deposition compared with the conclusions reached by Schröder and Pesch (2010) regarding other species sampled at a European level. Other investigators, who used analysis of variance (ANOVA) to analyze the data, found differences between *P. purum* and *Rhytidiadelphus squarrosus* (Arróniz-Crespo et al. 2008) and between *Pleurozium schreberi*, *Hylocomium splendens*, and *Dicranum polysetum* (Salemaa et al. 2008) in laboratory-based studies. However, the use of ANOVA is not appropriate for such comparisons because there is no independent variable and the investigators of the previous studies merely compared the concentrations of elements in two moss species in the field. Independent of the suitability of the test of comparison, the existence of interspecific differences in the concentrations of N are probably caused by the different bioaccumulation capacity of each species, which depends on the specific surface area, anion exchange capacity, and differences in morphology as suggested by Carballeira et al. (2008).

In contrast, there were also no interspecific differences in the concentrations of $\delta^{15}N$ between the two species under study. We are not aware of any field- or laboratory-based studies that have compared the $\delta^{15}N$ values in different species of moss. The results obtained in the present study allow us to state that there are no differences between *P. purum* and *H. cupressiforme* regarding isotope discrimination. The range of variation obtained for δ^{15}N values was much greater that that obtained for total N, which shows the greater sensitivity of the δ^{15}N signature. As a result, the coefficient of determination for δ^{15}N was twice that for total N (Fig. 3).

Total N values measured on a regional scale for *P. purum* varied between 0.60 and 2.40 %, i.e., a wider range than reported in the relevant literature (Table 1). For example, Solga et al. (2005) reported values between 0.71 and 1.94 % for the same species in a rural areas of Western Germany where and intensive agriculture livestock farming are common. The range is also slightly wider than that reported by Zechmeister et al. (2008) for various species of moss, including *P. purum*, in Austria (0.76 to 1.99 %). Likewise, the range was also slightly wider than that reported for epilithic mosses in Guiyang, one of the most highly contaminated cities in China (1.27–2.24 %) (Liu et al. 2008b). The δ^{15}N values ranged between −7.60 and −1.00 ‰ (Table 1) and were less negative than those reported by Solga et al. (2005) in Germany (−7.89 to −2.98 ‰), by Zechmeister et al. (2008) in Austria (−10.04 to −2.45 ‰), and even than those reported by Liu et al. (2008b) in China (−12.5 to −1.39 ‰). This indicates that there is greater deposition of oxidised N compounds in the area sampled in the present study than in the areas sampled in the other studies.

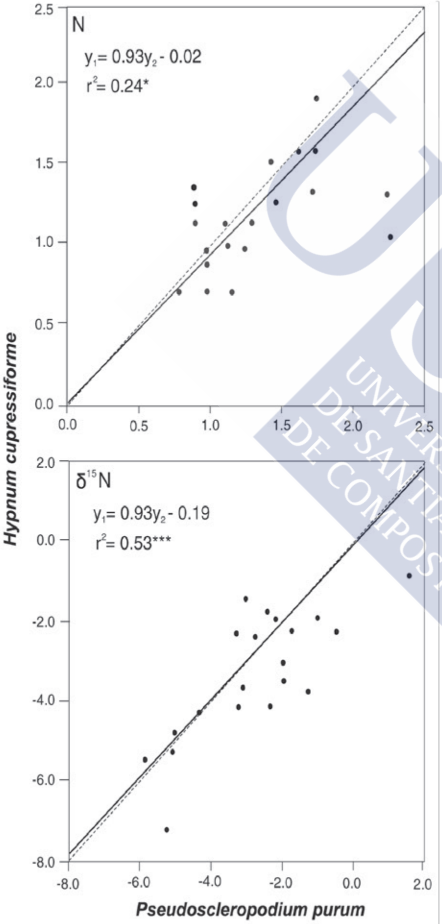


Fig. 3 Type II linear regressions between concentrations of N (%) and δ^{15}N (‰) determined in *P. purum* (y_2) and *H. cupressiforme* (y_1). The dashed lines correspond to the straight lines of slope equal to 1. The equation for the regression lines is included along with the value of the coefficient of determination (*$p \leq 0.05$ and ***$p \leq 0.001$)

After removing the only outlier in the total N data, the density distribution would be a symmetrical unimodal distribution (Fig. 2a) similar to that found for nutrients in *P. purum* (e.g., K in Pérez-Llamazares et al. 2011). However, the second mode observed on the left-hand side of the density distribution of δ^{15}N may reflect SSs that are contaminated by reduced forms of N. In terms of the presence of secondary modes, the polymodal distribution of δ^{15}N is rather similar to that reported by Aboal et al. (2006b) for heavy metals, such as cadmium (Cd), lead (Pb), and vanadium (V).

No spatial structure was detected in total N concentrations (Fig. 2a), and the values were distributed randomly throughout the region. The pattern observed for N in the moss is not consistent with the patterns observed in the deposition models (Fig. 1b, c). However, the results of the EMEP models should be considered cautiously because they are not constructed with immission or deposition data as previously mentioned. Moreover, the spatial resolution of these models is very low (50 × 50 km^2), which may lead to large errors, whereas the map that we have constructed includes 150 regularly distributed points.

However, spatial structure was detected in the δ15 N values, and the deterministic structure responsible for this was identified (Fig. 2c). As occurs with total N, the pattern of δ15 N in the moss in the region is not consistent with the N-deposition models (Fig. 1b, c). The most obvious conclusion on comparing the deposition-modelled δ15 N is that the values of δ15 N are highest (Fig. 2b) in the areas where deposition of oxidised N is lowest (southwest sector of the study area; Fig. 1c). The spatial structure of δ^{15}N explains a similar percentage of the raw data as that of metals, such as copper, Cd, and V, in the same study area (Aboal et al. 2006b). The most negative values of δ^{15}N were found in the centre of the study area, which is a largely agricultural area. The least negative values were observed in three zones: (1) the coastal area, which is the most populated and industrialized; (2) the southeastern zone, which is dominated by massifs and affected by two power plants located outside of the study region; and (3) the northern zone, which is influenced by two power plants in which the patterns of N deposition have previously been studied (Méndez et al. 2003), with results similar to those found in the present study.

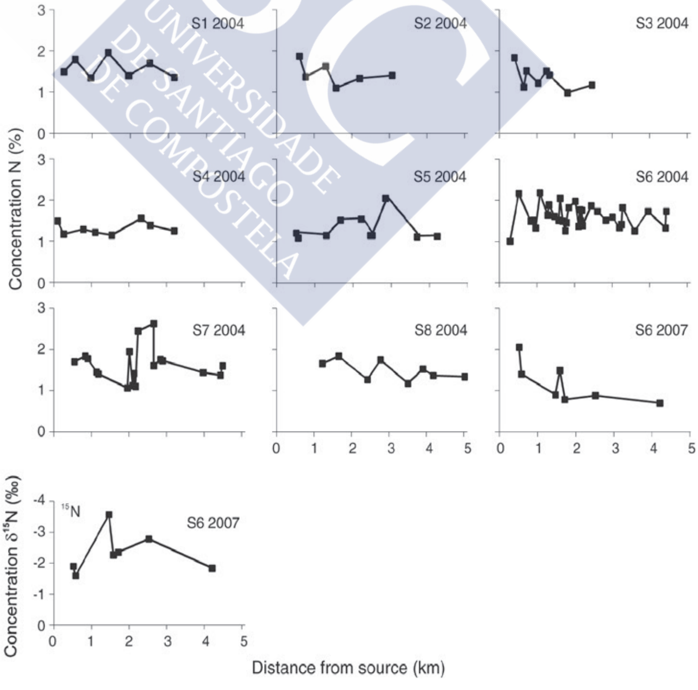
An exponential decrease in the concentrations of contaminants with increasing distance from the focal point of emissions, which has been observed for contaminants, such as heavy metals (Fernández et al. 2007), was not observed in the total concentration of N in the moss (Fig. 4). The only sector in which an exponential decrease in the concentrations of N was observed was sector no. 6 in 2007. Because this pattern was not observed in the other seven sectors, we may assume that the high concentration at the closest point to the smelter may have been due to random factors.

In contrast, most of the concentrations of N in moss samples from the surroundings of the smelter were between 1 and 2 %, which is on the right-hand side of the N distribution for the entire region (Fig. 2a), indicating that the moss in the area would be enriched in N. In four of the nine sectors sampled, the concentrations of N were >2 % in one or two samples. In sector no. 6 in 2007, the samples coincide with the point closest to the smelter. Sectors no. 4 through 6 are adjacent and located in the prevailing direction of the wind in this area during summer as shown in Fig. 2d. In a previous study performed in the surroundings of the same factory, in which concentrations of F were measured in *P. purum*, Real et al. (2003) indicated that this anion was mainly dispersed as a gas and it was deposited in the area equivalent to sectors no. 4 through 6. Because N is also an anion and the smelter emits about 1,770 ton of NO_x/NO_2 in one year, it is not surprising that greater concentrations of N were detected in the moss samples in this area. The findings for $\delta^{15}N$ are similar; the values occur on the left-hand side of the distribution and do not increase in relation to increasing distance from the smelter.

From the above, it appears that sources of atmospheric emission of N can be biomonitored by the measurement of $\delta^{15}N$ in terrestrial mosses, at least for large-scale pollution processes. In contrast, neither N nor $\delta^{15}N$ values were affected by the species of moss used, so both species can be used simultaneously in extensive surveys.

However, the percentage variance expressed by one species relative to another (Fig. 3) was much greater for $\delta^{15}N$ than for total N. In contrast, on studying the large-scale

Fig. 4 Relation between the values of N (%) and $\delta^{15}N$ (‰) in *P. purum* and the distance (km) from the source of contamination in the aluminium smelter for each of the areas sampled



patterns of N contamination (Fig. 2a, b), $\delta^{15}N$ analysis enables recognition of the regional pattern of the source of the N accumulated by the moss. Finally, regarding small-scale patterns of contamination, the information provided by $\delta^{15}N$ and by total N may be considered equivalent.

The different result obtained for $\delta^{15}N$ in large- and small-scale pollution processes may be related to the range of values detected. The range of $\delta^{15}N$ for the region varies between -7.60 and -1.00, and for the area around the smelter it varies between -3.57 and -1.59. The absence of deposition of reduced forms of N around the smelter narrows this range and explains why no decrease in $\delta^{15}N$ values was detected with increasing distance from the source.

N is metabolically important, unlike other contaminants that are often biomonitored by using terrestrial mosses (e.g., heavy metals, PAHs, etc.). Nitrogen is a macronutrient that affects the moss physiology. Several studies with bryophytes have shown that a greater availability of N results in an increase in the synthesis of amino acids and carbohydrates as well as changes in the photosynthetic rate and tissue concentrations of N (e.g., Koranda et al. 2007; Arróniz-Crespo et al. 2008; Liu et al. 2008c). One of the requisites of biomonitoring agents is that a linear response is maintained between the dose of an element to which the agent is exposed and the amount of the element that is bioconcentrated by the agent, which enables estimation of the deposition of the element received by the agent. However, Solga and Frahm (2006) showed (in a long-term laboratory experiment with diverse species of terrestrial moss) that this relationship is not linear and that N incorporated in moss tissues reaches a saturation point at realistic doses independent of the form in which N is supplied. Saturation of N accumulated in moss does not affect $\delta^{15}N$ because the rate of saturation is independent of the amount of N received. However, various investigators (e.g., Solga and Frahm 2006; Schröder and Pesch 2010; Stevens et al. 2011b) have indicated that bryophytes accumulate reduced forms of N more easily because their incorporation is less costly in metabolic terms due to the high cation exchange capacity of mosses. These differences would lead to isotope discrimination by mosses and preferential accumulation of the N^{14} isotope, mainly in ecosystems where N is not limiting. Thus, although the oxidised forms are present at greater concentrations than the reduced forms in the areas where deposition of both forms is highest (Fig. 1b, c), the reduced forms appear to be preferentially accumulated in the moss. The lowest values of deposition of oxidised and reduced forms of N (Fig. 1b, c) coincided with the least negative values of $\delta^{15}N$ (Fig. 2b) in the southwest zone of the region, which may be attributed to N limitation and greater deposition of the oxidised forms. As a consequence, the mosses would not reliably reflect the isotope composition of N deposition but would amplify N^{14}. Nevertheless, as shown in the present study, this would not be problematical for establishing the spatial patterns of the sources of N pollution.

Conclusion

In light of the findings of the present study, we recommend the measurement of $\delta^{15}N$ in terrestrial mosses to investigate atmospheric N deposition in preference to measurement of total N, at least in extensive surveys. First, although there were no interspecific differences in total N or $\delta^{15}N$ concentrations between *H. cupressiforme* and *P. purum*, $\delta^{15}N$ contents of both species were more closely related than those of total N. Second, measurement of $\delta^{15}N$ enables detection of large-scale spatial patterns of contamination generated by the sources of emission, unlike the measurement of total N.

The advantage of measuring $\delta^{15}N$ rather than total N in terrestrial bryophytes is that the former provides information about the source of N rather than the amount of N received and it is not strongly affected by metabolic regulation of this element or by saturation during accumulation. Furthermore, isotope discrimination appears to occur, with the bryophytes preferentially accumulating the N^{14} isotope. Although this amplifies the signal of reduced forms, this is not problematical in terms of determining spatial patterns of distribution.

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CHAPTER 03


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Use of a moss biomonitorinf method to compile emission inventories for small-scale industries.

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Abstract We used a method of detecting small scale pollution sources (DSSP) that involves measurement of the concentrations of elements in moss tissues, with the following aims: i) to determine any common patterns of contaminant emissions for individual industrial sectors, ii) to compare any such patterns with previously described patterns, and iii) to compile an inventory of the heavy metals and metalloids emitted by the industries considered. Cluster analysis revealed that there were no common patterns of emission associated with the industrial sectors, probably because of differences in production processes and in the types of fuel and raw materials. However, when these variables were shared by different factories, the concentrations of the elements in moss tissues enabled the factories to be grouped according to their emissions. We compiled a list of the heavy metals and metalloids emitted by the factories under study and found that the DSSP method was satisfactory for this purpose in most cases (53 of 56). The method appears to be a useful tool for compiling contaminant inventories; it may also be useful for determining the efficacy of technical improvements aimed at reducing the industrial emission of contaminants and could be incorporated in environmental monitoring and control programmes.

Keywords: Air quality; Cluster analysis; Heavy metals; *Pseudoscleropodium purum*

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1.Introduction

The high degree of industrialization that has taken place in the last century has led to increased emissions of contaminants to the atmosphere. This has led to the search for methods of characterizing and monitoring such emissions, with the aim of minimizing the environmental impact of industrial processes. Biomonitoring of heavy metals and metalloids with terrestrial mosses is recognised as a suitable tool for this type of control. Atmospheric emission of contaminants can affect small or large areas of land, depending on the source of contamination. Emissions derived from large factories, densely populated areas and agricultural activities generate large-scale contamination processes. Emissions derived from small and medium-sized factories generate small-scale contamination processes. To date, research in which bryophytes are used to study atmospheric contamination by heavy metals and metalloids has addressed both large- and small-scale contamination. Studies of large-scale emissions have focused on evaluating patterns of contamination in sampling networks previously established for large areas, at densities of approximately 1.5 sampling sites 1000 km-2 [1]. Research involving small scale contamination has centred on sources of emission that affect restricted areas [2-5], with the sampling effort involving more than 1 sampling site 1 km$^{-2.}$ However, study of the contaminants produced by various dispersed sources of emission that each generates small-scale contamination over a large area is complicated. When a low density of sampling sites is used in a large area, many of the emission sources may be missed if there are no sampling sites nearby [6]. However, studies involving high densities of sampling sites aimed at capturing each dispersed source of contamination are not viable from economic or operational points of view.

One possible solution to this problem is to use the method proposed by [7] for detecting small-scale pollution sources (DSSP). This method enables the contaminants emitted by such sources to be detected by using a very small number samples. The method is based on the shape of the curves that relate the concentrations of heavy metals and metalloids in moss tissues

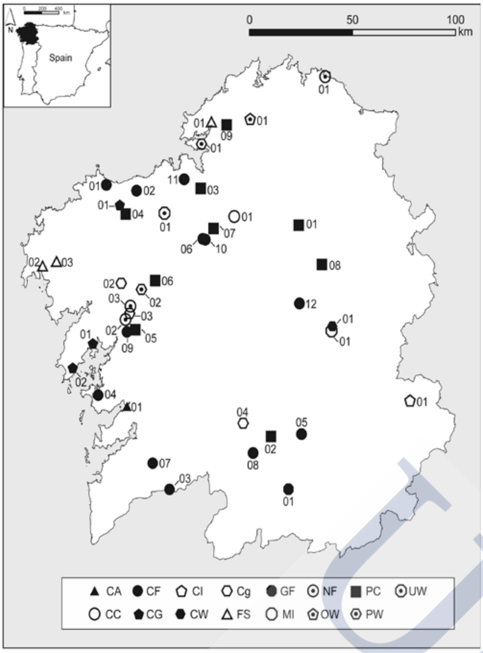

Fig 1. Location of the moss *P. purum* SS in the surroundings of the different factories classified in several industrial sectors (see legend for Table 1).

to the distance from the source of emission [2-3, 8-12]. According to this relationship, there will be a large difference between the concentrations of the contaminants in a sample of moss collected from close to a source of contamination and in another collected at some distance from the source. However, it is essential to confirm that the difference is actually due to contaminant emissions and not simply to the natural variability in the concentrations of elements in the moss. To ensure this, we can use reference distributions of the differences in the concentrations of each element established using paired sampling points in uncontaminated areas [7]. If the observed differences do not belong to the reference distributions, we can infer that the source is contaminating the surroundings.

The DSSP method is a useful tool for compiling emission inventories such as the European Pollutant Release and Transfer Register (E-PRTR; http://prtr.ec.europa.eu/). This register implements the UNECE (United Nations Economic Commission for Europe) PRTR Protocol of the Aarhus Convention to facilitate access to information about the contamination emitted by the main industrial processing plants in the European Union, Iceland, Norway, Lichtenstein, Serbia and Switzerland. Thus, it includes contaminant emissions to the atmosphere, water and soil of approximately 28,000 factories included in 65 different sectors. However, in most cases the available data are restricted to estimated or calculated values as the number of factories involved is so large that it is not feasible to measure all of the emissions directly. Moreover, in compiling this type of official register and in previous studies of the presence of contaminants in the environment, researchers have tended to associate the emission of certain types of contaminants with particular industrial processes and contaminants, without considering whether all industrial plants dedicated to the same activity emit the same contaminants. The DSSP method may therefore be useful for characterizing the emissions from different types of industries and comparing the results with the data included in the official registers.

However, there are certain limitations to the proposed method, as the following conditions must be fulfilled for correct application of the technique: i) each source of contamination must be isolated from other possible sources; ii) the distance between the closest sampling site and the source of contamination must be small enough to capture the contamination gradient; iii) the moss samples must represent the temporal variability inherent in the concentrations of contaminants in moss [13,14]; and iv) the sampling sites must be arranged in the direction in which the decrease in the concentrations in the moss is most evident. If the above conditions are not fulfilled, the method will not be capable of detecting the existence of contamination (false negatives), although it will not detect contamination that does not exist (false positives).

The main aims of the present study were as follows: i) to determine any common patterns of contaminant emissions for individual industrial sectors, ii) to compare any such patterns with previously described patterns, and iii) to compile an inventory of the heavy metals and metalloids emitted by the industries considered.

2. Material and Methods

2.1.Sampling

Samples of *Pseudoscleropodium purum* (Hedw.) M. Fleisch were collected in Galicia (NW Spain), between 1999-2008, at sampling sites (SS) in the surroundings of 43 different factories (some were sampled during the course of several years) that are potential sources of contamination. The factories represent 26% of the factories in the region that are included in the E-PRTR, and they are categorized into 14 industrial sectors: chlor-alkali plants (CA),

Table 1. Information about the factories under study. The name of each industrial sector to which the factories belong is indicated using the following abbreviations: chlor-alkali plants (CA), ceramic factories (CF), cogeneration plants (CG), chemical industries (CI), chemical industries with cogeneration (CC), cement works (CW), ferrous-smelters (FS), glass factories (GF), mineral industries (MI), non-ferrous smelters (NF), waste oil treatment (OW), paper and wood production with cogeneration (PC), paper and wood production (PW), urban waste incinerators (UW)); production: annual production (1: t year^{-1}; 2: m^3 year^{-1}). Source of production data: a: Regional Goverment (Xunta de Galicia, www.xunta.es); b: E-PRTR, c: business website; n.a.: not available.

Industry	Year	Distance from focal point of emission(m)	Product/type of industry	Production
CA01	99; 07	267	Chlor-alkali and derived products	33,552^{1,a}
CF01	06	173	Bricks	70,000^{1,a}
CF02	06	472	Bricks and tiles	>20,000^{1,b}
CF03	06	210	Bricks and filler blocks	>20,000^{1,b}
CF04	06	461	Bricks and tiles	>20,000^{1,b}
CF05	06	224	Ceramics, Tiles, Porcelain	>20,000^{1,b}
CF06	07	208	Bricks, Thermo-clay blocks	29,000^{1,b}
CF07	07	131	Bricks and tiles	>20,000^{1,b}
CF08	07	176	Bricks	>20,000^{1,b}
CF09	07	131	Bricks and tiles	90,000^{1,c}
CF10	08	119	Tiles	63,985^{1,b}
CF11	08	147	Bricks	>20,000^{1,b}
CF12	08	120	Bricks	>20,000^{1,b}
CG01	07	172	Cannery and cogeneration	23,000^{1,b}
CG02	08	110	Cannery and cogeneration	>54,000^{1,b}
CG03	08	242	Animal feed and cogeneration	140,000^{1,b}
CG04	08	193	Aviculture and cogeneration	141,000^{1,b}
CG05	07	403	Cannery and cogeneration	>20,000^{1,c}
CG06	08	298	Cannery and cogeneration	60.000^{1,a}
CI01	07	1332	Electrolytic coatings	>36,000^{1,c}
CC01	07	502	Bioethanol	138,723^{2,b}
CW01	07	317	Cement and clinker	369,910^{1,b}
FS01	04; 05; 06; 07	334	Iron and steel	750,000^{1,b}
FS02	06; 07	444	Silicon and manganese	n.d.
FS03	02; 07	387	Silicon and manganese	n.d.
GF01	07	331	Glass, glassfibre	>6,000^{1,b}
MI01	07	220	Mineral products	>134,500^{1,b}
NF01	99; 04; 07	922	Aluminium, Alumina and derivatives.	n.d.
NF02	07	288	Aluminium profiles for industrial use	81,500^{1,c}
NF03	07	281	Aluminium profiles for industrial use	70,000^{1,c}
OW01	07	208	Reutilization of oil	>3,000^{1,b}
PC01	07	317	Wood boards	184,032^{2,b}
PC02	07	235	Wood boards	184,523^{2,b}
PC03	07	248	Wood boards	>5,500^{2,b}
PC04	07	267	Wood boards	n.d.
PC05	07	348	Wood boards	n.d.
PC06	07	408	Wood boards	318,873^{2,b}
PC07	07	209	Wood boards	n.d.
PC08	07	181	Wood boards	135,000^{1,b}
PC09	07	392	Wood boards	n.d.
PW01	07	585	Glues	43,990^{1,b}
PW02	07	237	Paper and cardboard	23,315^{1,b}
UW01	00; 02; 03	1393	Urban waste incineration	541,759^{1,b}

ceramic factories (CF), cogeneration plants (CG), chemical industries (CI), chemical industries with cogeneration (CC), cement works (CW), ferrous-smelters (FS), glass factories (GF), mineral industries (MI), non-ferrous smelters (NF), waste oil treatment (OW), paper and wood productions with cogeneration (PC), paper and wood productions (PW) and urban waste incinerators (UW) (Fig 1)

Table 1 includes detailed information about the factories studied, including the industrial sector, year in which sampling was carried out, distance of sampling point from the focal point of emissions, the products manufactured and the annual production.

The samples of moss were collected following the method proposed by [7]. Briefly, the SS were arranged along two polar radii from the focal point of emission. One transect followed the direction of the prevailing wind in Galicia (NE-SW) and the other was arranged perpendicular to this (NW-SE), with the aim of detecting any anisotropy in the dispersal of the contaminants. Samples were collected from two SS on each radius; one of the SS was located as close as possible to the focal point of emission and the other at a distance of between 970 and 1250 m from the first, as recommended in the method (Table 1). Whenever possible, the samples were collected in open areas or small clearings in forests, although not next to trees, and areas close to main roads and centres of population were avoided [15]. At each SS, approximately 30 subsamples of similar weight were combined to make a single composite sample. Whenever possible, the dimensions of the collection area were between 20 x 20 m and 50 x 50 m [16-18].

Table 2: Probability that the difference in the concentrations of the different elements in samples of the moss P.purum collected in the surroundings of different factories belong the reference distribution corresponding to uncontaminated areas (see details in text). 0= not significant, 1= p<0.05, 2= p<0.01, 3 = p<0.001. Industry codes are as listed in Table 1; the number after some codes corresponds to the sampling year, the letters after some years correspond to the sampling months (j: January, m: May, s: September, o: October, n: November).

	Elements																
Industry	Al	As	Ba	Be	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Se	Sr	V	Zn
CA01.99j	3	0	0	3	3	0	0	2	3	3	0	3	3	0	0	3	2
CA01.99s	3	0	0	3	3	0	0	3	3	3	0	3	3	0	2	3	3
CA01.07	3	2	0	0	0	2	3	2	3	3	0	0	3	0	0	3	0
CF01	0	0	0	0	0	0	3	0	0	0	0	3	0	3	0	3	0
CF02	3	0	3	0	2	3	3	0	3	0	0	3	0	2	1	3	0
CF03	0	0	0	0	0	3	0	2	0	0	0	3	0	2	0	3	3
CF04	3	0	0	3	0	0	3	0	1	0	0	3	0	0	0	3	0
CF05	0	0	2	0	0	0	0	0	0	0	0	0	0	0	1	3	0
CF06	0	0	1	2	0	0	0	1	0	3	0	0	0	3	0	3	3
CF07	1	3	2	0	0	1	0	2	3	2	0	2	3	3	0	3	3
CF08	2	3	3	3	2	3	1	1	3	0	0	3	3	0	0	3	3
CF09	3	3	3	3	3	3	1	1	3	0	0	2	3	1	1	3	2
CF10	3	3	3	3	0	0	3	3	3	2	0	3	3	0	0	3	1
CF11	3	3	3	3	0	3	0	0	3	0	0	0	2	0	2	3	0
CF12	3	2	2	3	0	0	0	0	3	0	0	1	3	0	0	3	0
CG01	0	3	1	3	3	3	3	0	3	0	0	3	0	0	2	3	0
CG02	3	0	0	3	1	3	0	0	2	0	0	0	0	0	0	3	1
CG03	3	0	3	3	1	0	3	0	3	0	2	0	3	0	2	3	0
CG04	3	3	0	3	0	3	0	0	3	0	0	3	0	0	0	3	0
CG05	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	3	0
CG06	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0
CI01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC01	0	0	0	0	2	0	0	0	0	0	0	3	0	0	0	0	0
CW01	3	3	1	0	2	3	0	0	3	0	0	0	3	0	0	3	0
FS01.04j	0	0	2	3	3	3	3	3	3	3	0	3	3	0	0	2	3
FS01.04o	0	0	3	0	3	3	0	3	3	3	0	3	3	0	0	3	3
FS01.05	1	0	1	0	3	3	3	3	3	3	0	3	3	0	0	3	3
FS01.06	1	0	1	0	3	3	1	3	3	3	0	3	3	0	0	3	3
FS01.07	0	0	2	0	3	3	0	3	3	3	0	3	3	0	0	3	3
FS02.06	3	0	0	3	3	3	3	0	3	2	3	3	3	0	0	2	0
FS02.07	3	0	3	3	3	3	1	1	3	0	3	3	3	0	0	2	0
FS03.02	0	3	1	0	0	3	3	3	3	0	0	3	3	0	0	3	3
FS03.07	0	0	0	0	0	0	0	3	1	0	0	1	0	0	0	0	0
GF01	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
MI01	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	3	0
NF01.99m	3	0	1	2	3	3	0	3	3	3	0	3	3	0	0	3	3
NF01.99n	1	0	0	0	0	0	0	3	0	0	0	0	0	0	2	3	3
NF01.04	3	0	1	0	3	0	0	0	2	0	0	3	3	0	0	3	3
NF01.07	3	0	0	1	3	3	0	0	2	3	0	3	3	0	0	3	0
NF02	2	0	3	0	0	0	0	2	0	3	0	0	0	0	1	0	3
NF03	3	0	1	3	3	2	0	2	3	0	0	0	1	0	2	3	0
OW01	3	0	0	2	3	3	3	0	3	0	0	3	3	0	0	0	3
PW01	0	0	0	1	0	2	3	0	0	3	0	0	0	3	0	0	0
PW02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PC01	3	0	0	1	1	0	0	0	2	0	0	0	3	3	0	2	0
PC02	3	3	3	3	0	3	0	2	3	0	0	0	3	0	1	3	0
PC03	3	3	3	3	0	3	3	0	3	0	0	2	3	1	1	3	3
PC04	0	3	0	3	0	0	0	0	3	0	0	0	3	0	0	3	0
PC05	0	0	0	0	0	0	0	0	0	0	0	1	3	3	0	0	0
PC06	0	3	3	0	0	0	3	0	0	0	0	0	2	1	0	2	0
PC07	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	1	0
PC08	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
PC09	3	3	1	3	0	3	3	1	3	0	0	3	3	1	1	3	0
UW01.00	0	0	2	0	3	0	0	0	0	0	0	0	0	0	3	0	0
UW01.02	0	0	0	0	1	0	0	0	0	0	0	0	0	0	3	0	0
UW01.03	3	0	3	3	3	3	0	1	3	0	3	3	3	0	2	2	0

2.2.Chemical analysis

Prior to chemical analysis, the samples were cleaned to remove adhered plant remains, and apical segments of moss (3-4 cm lon) were separated from the shoots. The samples were homogenized in an ultracentrifugal mill (Retsch ZM200 metal free, Retsch GmbH) and digested with HNO_3 (65% analytical grade) in a microwave oven (CEM MDS2100). The concentrations of Al, Cu, Fe, Mn and Zn were determined by flame atomic absorption spectrometry (Perkin Elmer 2100). The concentrations of Ba, Be, Cd, Co, Cr, Ni, Pb, Sr and V in the moss tissues were determined by graphite furnace atomic absorption spectrometry (Perkin Elmer Analyst 600). As was determined by atomic fluorescence spectrophotometry (PSA Excalibur). Finally, the Hg in the powdered moss samples was determined directly in an elemental analyzer (DMA 80 Milestone).

Two aliquots of the same sample were analyzed once every nine samples, to control the analytical quality. Certified reference material (M2, *Pleurozium schereberi*; [19]) was also analyzed, once every nine samples, to control the extraction and analytical procedures. The percentage recovery of the elements ranged between 80% and 125% (except for As and Be, for which it was approximately 40%). The elements in all samples were above the quantification limit, and the errors associated with the determination [20] ranged between 1-12%. Finally, the existence of contaminating material was controlled for by using analytical blanks, one every nine samples.

2.3. Statistical analysis

The differences in concentrations between the points along each sampling radius were calculated. These were then compared with the reference distributions for unpolluted areas to determine the probability of obtaining such values. Differences for which $p<0.01$ were considered to indicate pollution and were coded as 1 (otherwise, 0), and a presence-absence matrix of 56 samples x 17 elements was constructed. The matrix was then analyzed by cluster analysis to determine the similarity between samples, by applying Ward's [21] agglomerative algorithm and Jaccard's [22] distance. All analyses were carried out with R statistical software [23].

3. Results

For each element and factory studied, the lowest probability that the two sampling transects were included in the corresponding reference distribution is shown in Table 2. Although factories such as CI01 and PW02 did not emit any contaminants ($p>0.01$), others such as CF07, CF08, CF09, CF10, FS01, NF01, PC03, PC09 and UW01.03 emitted between 12 and 15 of the 17 elements studied.

The number of factories in each industrial sector that emitted the elements under study in relation to the concentrations of the elements in the moss samples are shown in Table 3. The elements emitted by each industrial sector, according to the E-PRTR and [24] are also shown in Table 3. The data provided by the E-PRTR refer to each individual factory and they were grouped to yield representative data for each sector. Comparison of the three estimates reveals some discrepancies. Thus, the DSSP method detected contamination in industrial sectors in which the other two methods did not detect any contamination; this was observed for all of the elements considered in the study. The opposite was also observed, *i.e.* the DSSP did not detect contamination in some cases where it was indicated by the other two methods. The results of the cluster analysis used to group the different industries under study (Fig. 2) revealed two well differentiated clusters, in which A represents the industries that emit very few elements (*i.e.* between 1 and 6, with the exception of CF07, which emits 10 different elements) and B includes those industries that emit 9 or more elements. No emission of contaminants was detected in the surroundings of 5 of the factories (CI01, GF01, PC07,PC08 and PW02) ($p>0.01$).

4. Discussion

4.1 Characterization of the contaminants emitted by the different industrial sectors

The cluster analysis clearly showed that there was no common pattern of emission for the individual industrial sectors. This may be due to the existence of false negatives produced by the DSSP for some factories but not others, although the degree of consistency indicates that the results are reliable. For example, the ceramics factories CF06, CF03 and CF07 are grouped in the same cluster as they all contaminate the surroundings with Cu, Se, V and Zn (Table 2). The raw materials used in these factories are probably very similar. For the cogeneration plants, two clusters were identified: Cluster A included CG05 and CG06, and Cluster B included CG02, CG04 and CG01. The factories in Cluster A both use high grade fuel and only contaminate their surroundings with V, whereas the factories in Cluster B contaminate with Be, Co, Fe and V, emissions of which are associated with fuel combustion (Table 3), so that the use of the same type of fuel again explains the grouping. In the paper and wood production sector, factores PC06, PC01 and PC05 are grouped together. These three factories belong to the same company and use the same type of fuel (and treatments such as filtration),raw materials used in these factories are probably very similar. For the cogeneration plants, two clusters were identified: Cluster A included CG05 and CG06, and Cluster B included CG02, CG04 and CG01. The factories in Cluster A both use high grade fuel and only contaminate their surroundings with V, whereas the factories in Cluster B contaminate with Be, Co, Fe and V, emissions of which are associated with fuel combustion (Table 3), so that the use of the same type of fuel again explains the grouping. In the paper and wood production sector, factories PC06, PC01 and PC05 are grouped together. These three factories belong to the same company and use the same type of fuel (and treatments such as filtration), raw materials and production process (pers. comm.), and therefore should perhaps be expected to display the same pattern of emission with Pb, Se and V. The same company owns another factory, PC02, which is not grouped with the others. However, this factory is located in an industrial estate where the proximity of other factories makes it impossible to detect the individual effects of each. For the other factories belonging to the same industrial sector, no common patterns of emission were observed. Although it may appear logical to assume that the factories involved in the same activity will emit the same contaminants to the atmosphere, differences in the type of fuel, raw materials and productive processes can lead to different patterns of emission.

Finally, comparison of the data obtained by the DSSP method with the data included in the E-PRTR and the data compiled by [24] revealed some discrepancies, as already mentioned. The advantage of the DSSP method is that it is not based on theoretical assumptions about the functioning of the industrial processes involved (as in the E-PRTR) or on data corresponding to other factories that may be very different from those under study here. Different factories may use different internal procedures (*e.g.* cogeneration of energy) or different grades of raw material (fuel or other combustible material) that will lead to differences in the emissions. Although such differences between factories are very difficult to reflect with the other two methods, the DSSP method is based on the real situation in the surroundings of each factory. One great advantage of this method is that no prior assumptions are made.

4.2 Study Limitations

This is the first study in which the DSSP method has been used to obtain an inventory of the metals and metalloids that are emitted over a large area by different factories involved in different industrial sectors. The experience acquired has enabled us to identify a series of limitations to the application of the method.

First, each of the factories being monitored must be isolated from other possible sources of contamination (*e.g.* from other factories, roads or densely populated areas). This is the only way of ensuring that the differences in concentrations of the elements in the moss samples are definitely related to the emissions from each factory under consideration. This requisite was not fulfilled in each of the cases studied (*i.e.* CA01, PC02 and UW01), for the following reasons: i) factory CA01 is situated beside a cellulose pulp factory, a concrete factory and a busy road; ii) factory PC02 is located on an industrial estate; and iii) factory UW01 is located at a distance of 5 km from a power plant. Thus, the metals and metalloids in the moss samples around these factories may be derived from different sources that are impossible to distinguish. Moreover, the required criterion of a pattern of decrease in concentrations with increasing distance from the source will not be fulfilled if the more distant samples receive inputs from other sources, and false negative results may be produced in cases i and ii. However, factory UW01 is located close to a source of contamination that emits large-sale contamination and this problem will not occur (*i.e.* false negative results). This type of source generates gentle gradients that affect large areas, which at the scale used (*i.e.* approx. 1 km) are negligible in comparison with those generated by small-scale sources. For example, densely populated areas in the study region act as diffuse sources of Pb pollution and create gradients that affect the entire area (*i.e.* 8.5 ng g^{-1} Pb km^{-1}, [6]).

The second problem identified in association with DSSP method is the distance between the first sampling site and the possible source of emission. In this respect, if the first SS is too far from the factory, the contamination gradient will not be detected and the possible source of emission will be overlooked. With a few exceptions, the distance between the first SS on each sampling transect and each source of emission was sufficiently small (100 - 400 m) to prevent this problem (Table 1). Only at CI01, NF01 and UW01, the distance was much greater (up to 1000 m). Owing to the large size of the NF01 and UW01 facilities, which extend hundreds of metres from the focal point of the emissions (*i.e.* chimneys), it was impossible to place the SS close to the source of emission. However, the gradient of contamination also extended further than for other sources, so that the contaminants were detected in the moss

Table 3. Elements emitted by each industrial sector (see legend for Table 1), according to the concentrations in moss, according to the European Pollutant Release and Transfer Register (E-PRTR) and according to Merian et al. (2004). n: number of factories in each industrial sector. Elements associated with fuel combustion are shown in brackets. The elements identified by the moss technique and in the E-PRTR inventory are shown in bold type. The elements identified by the moss technique and by Merian et al. (2004) are indicated in italics:

Sector	n	Elements																	E-PRTR inventory	Merian *et al*. (2004)
		Al	As	Ba	Be	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Se	Sr	V	Zn		
CA	1	1	1	-	1	1	1	1	1	1	***1***	-	1	1	-	1	1	1	**Hg**	*Hg*
CF	12	*7*	***6***	*8*	*7*	*3*	*5*	4	3	7	*3*	-	***8***	*6*	5	1	*12*	5	**Ni**, **As**	*Ba, Co, Ni (Al, As, Be, Cd, Co, Hg, Ni, Pb, V)*
CG	6	*3*	*2*	1	*4*	*1*	*3*	2	-	4	-	1	***2***	*1*	-	2	*6*	-	**Ni**	(*Al*, *As*, *Be*, Cd, *Co*, Hg, Ni, *Pb, V*)
CI	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CC	1	-	-	-	-	1	-	-	-	-	-	-	1	-	-	-	-	-	-	-
CW	1	*1*	*1*	-	-	*1*	*1*	-	-	1	-	-	-	*1*	-	-	*1*	-	-	*Al* (*Al*, *As*, Be, *Cd*, *Co*, Hg, Ni, *Pb*, *V*)
FS	3	1	*1*	2	2	*2*	*3*	*3*	**2**	*3*	2	1	*3*	***3***	-	-	*3*	***2***	**Cu**, **Pb**, **Zn**	*As ,Cd, Co, Cr, Fe, Ni, Pb, V, Zn*
GF	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Cr, Ni, Pb	As, Co, Ni
MI	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	Ni
NF	3	*3*	-	1	*2*	*2*	*2*	-	*3*	2	***2***	-	***1***	*1*	-	2	*2*	*2*	**Hg**, **Ni**	*Al, Cd, Co, Cu, Hg, Ni, Zn (Al*, As, *Be, Cd, Co, Hg, Ni, Pb, V)*
OW	1	1	-	-	1	1	1	1	-	1	-	-	1	*1*	-	-	-	*1*	-	*Pb*, *Zn*
PC	9	*4*	***5***	3	*4*	-	*3*	*3*	1	5	-	-	***2***	*7*	2	-	*6*	**1**	**As**, Cd, Hg, **Ni, Zn**	*As*, *Cr* (*Al*, *As*, *Be*, Cd, *Co*, Hg, *Ni, Pb, V*)
PW	2	-	-	-	-	-	1	*1*	-	-	1	-	-	-	1	-	-	-	Ni	As, *Cr*
UW	1	*1*	-	1	*1*	1	1	-	-	1	-	1	*1*	*1*	-	1	1	-	Hg	*Al*, *Be*, Hg, *Ni*, *Pb*

samples. However, other factors prevented the first SS being located any closer to the source of emission in factory CI01: i) the dense population in the surrounding (urban) area, ii) the orography of the area, and iii) the Mediterranean climate, which is not favourable for growth of the moss species used. Therefore, no contamination gradient was detected at factory CI01, which, unlike the other factories, is a small facility with a low chimney that emits contaminants over a relatively short distance. When under circumstances such as these prevent growth of *P. purum* in the surroundings of the factory under study, moss transplants can be used instead of native moss, although this will require recalibration of the method.

Third, the high temporal variability in the concentrations of metals and metalloids in the moss species used in the method must be taken into account [13, 14]. This variability may be due to environmental characteristics and to physicochemical and biological processes that affect the moss. However, despite the variability, the results obtained for the factories that were monitored at different times were consistent. For example, samples were obtained in the surroundings of factory FS01 on 5 occasions between 2004 and 2007, and Ba, Cd, Co Cu, Fe, Hg, Ni, Pb, V and Zn were detected on all occasions (Table 2).

The differences observed over time for CA01, FS03 and UW01 can be explained by changes in the emissions. In the case of factory CA01, on both occasions in which sampling was carried out in 1999, the factory contaminated the surroundings with the same metals (*i.e.* Al, Be, Cd, Cu, Fe, Hg, Ni, Pb, V and Zn; Table 2). However, in 2007, contamination by Cd, Ni and V was not detected, although contamination by As, Co and Cr was detected. This change may have been due to the implementation of technological improvements in the factory (or in the other potential sources in the surroundings) and or the reduction in the production level. Thus, in 2002, factory FS03, which produces ferrosilicon, was identified as a source of contamination by As, Ba, Co, Cr, Cu, Fe, Ni, Pb, V and Zn (Table 2). However, after the installation in 2006 of a fume hood to capture secondary microsilica by a dry filtration method, the only elements detected in 2007 were Cu, Fe and Ni. Finally, during the construction of factory UW01 in 2000, contamination by Ba, Cd and Sr was detected in the surrounding area (Table 2). In 2002, two years after the factory became operational, only Cd and V were detected, although once production reached maximal levels, contamination by Al, Ba, Be, Cd, Co, Fe, Mn, Ni, Pb, Sr and V was detected. The variation in the results obtained for factories FS02 and NF01 at the different sampling times may also be due to emissions; however there is not sufficient information available to enable interpretation of the results.The method can also be used to monitor the efficacy of technical improvements used in factories with the aim of reducing contamination (*e.g.* FS03) and in designing programmes to monitor the surrounding environments before and after activities (*e.g.* UW01).

5. Final remarks and conclusions

In general, we did not identify any common patterns of contamination for the groups of factories in the same industrial sector. This may be due to differences in the types of fuel and the composition of the raw materials used, as well as to differen-

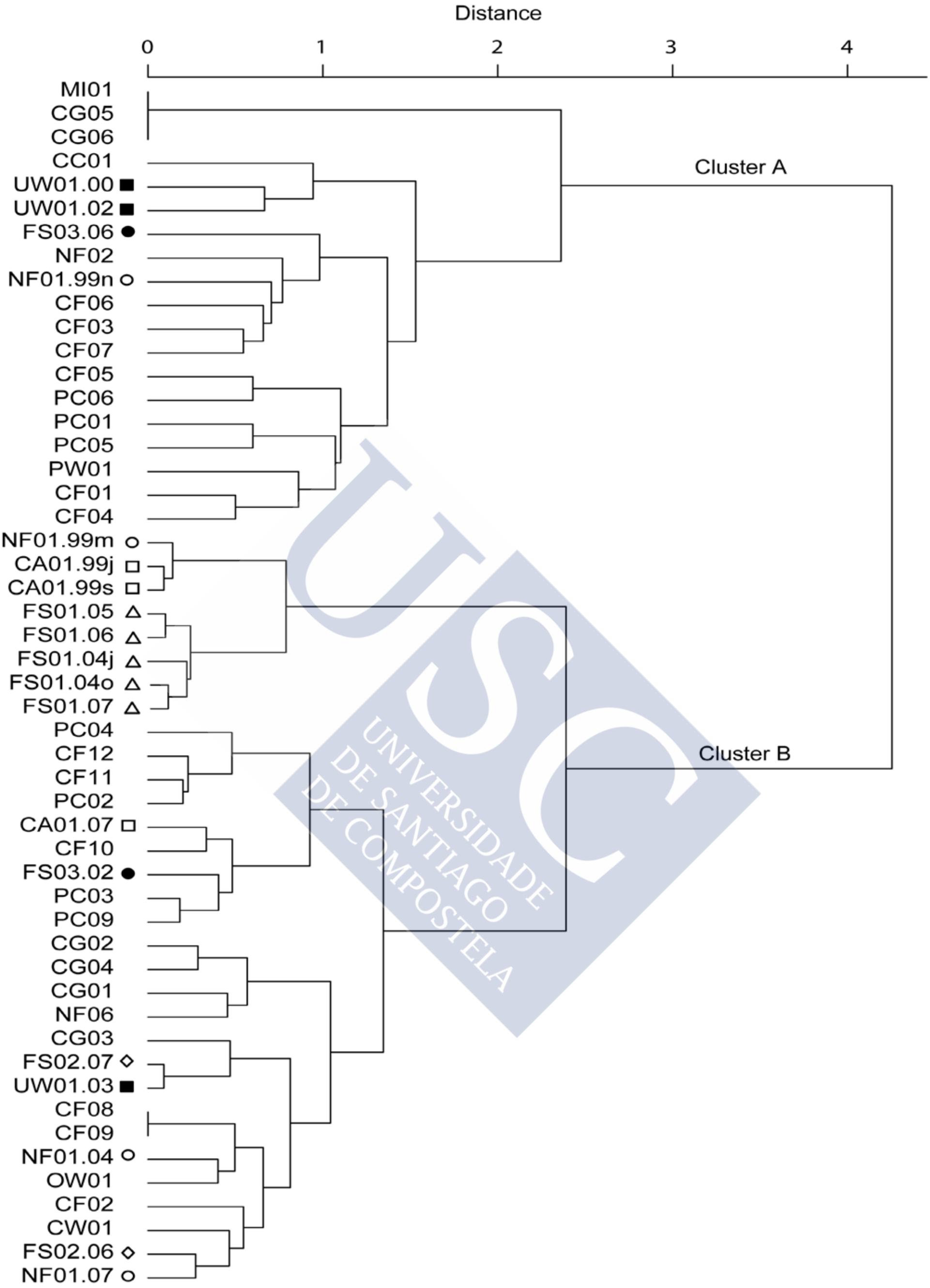

Fig 2. Cluster of the 43 factories belonging to the different industrial sectors (see legend for Table 1). Factory codes are the same as in Table 1, the number after some codes corresponds to the sampling year, the letters after some years correspond to the sampling months (j: January, m: May, s: September, o: October, n: November).

ces in the machinery, productive processes and the work practices. However, when these variables are shared by factories, measurement of the concentrations of elements in moss samples enabled the factories to be grouped according to sector.

The DSSP method enabled compilation of data for inclusion in an inventory of the heavy metals and metalloids released to the atmosphere by different industries.The conditions at the factory and surroundings hampered correct use of the method in only 3 of 56 cases. Furthermore, the DSSP proved to be a good alternative to the estimates made in published studies and the model estimates used in the E-PRTR inventory, with the advantage that it does not yield false positive results in the identification of contaminants emitted by factories. It is also a simple and accurate method for monitoring emissions and evaluating the efficacy of the technical improvements implemented in factories. It is also a simple and accurate method for monitoring emissions and evaluating the efficacy of the technical improvements implemented in factories.

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CHAPTER 04


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Influence of the physicochemical characteristics of pollutants on their uptake in moss.

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Abstract Bryophytes are commonly used as biomonitors to estimate the atmospheric deposition of heavy metals and metalloids. However, the tissue concentrations of these elements in moss do not always accurately reflect atmospheric levels. The aim of the present study was to investigate whether element uptake in moss is affected by the physicochemical characteristics of the elements. Factor analysis was used to identify any patterns of covariance in the accumulation of elements in samples of the moss *Pseudoscleropodium purum* collected from the surroundings of different factories and from control sites. The variation in the concentrations of elements was similar in moss from both types of sites and was related to the binding properties of the elements. This suggests that the physicochemical characteristics of the elements determine the uptake of metals and metalloids from the atmosphere. Therefore, in studies that use multiple correlations among elements as indicators of a common origin of contaminants, erroneous conclusions may be reached by overlooking the adsorption properties of the moss.

Keywords: Biomonitoring; Factor Analysis; Heavy metals;*Pseudoscleropodium purum*

1. Introduction

Since the moss biomonitoring technique was first reported (Rühling and Tyler, 1968), bryophytes have been widely used as biomonitors to estimate the atmospheric deposition of heavy metals and metalloids. However, the concentrations of elements in moss samples do not always accurately reflect the atmospheric deposition of these elements (Aboal *et al.*, 2010): the tissue burdens will not only depend on the amount of a particular contaminant in the atmosphere, but also on the physicochemical characteristics of the contaminants and the uptake processes.

Although numerous biomonitoring studies have been carried out with moss, relatively little is known about the specific physicochemical mechanisms involved in the bioadsorption processes, in comparison with those in other organisms (algae, bacteria, fungus, plants; González and Pokrovsky, 2014). In fact, there are no reports in the available literature about how the physicochemical characteristics of contaminants and of the uptake processes in mosses might affect the bioconcentration of different atmospheric contaminants. Aspects such as the amount of a contaminant emitted by factory and the type of emission (gaseous or particulate, particle size, etc.) may have important effects on the uptake process. The physicochemical processes in mosses include aspects such as the affinity of contaminants for cation exchange sites (Nieboer and Richardson, 1980), competition by metals or metalloids for cation exchange sites (Couto *et al.*, 2004) and synergistic uptake of contaminants (Sun *et al.*, 2009).

Therefore, the objective of the present study was to establish the extent to which the physicochemical characteristics of elements determine their uptake in mosses. Factor analysis was carried out with the aim of identifying any patterns of covariance in the bioconcentration of elements in moss samples collected from the surroundings of different factories and from control sites. The working

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hypothesis of the study is that as these types of samples are subjected to different levels and types of contaminants, different patterns will be observed if caused directly by the inputs of atmospheric contaminants, but that if there is some underlying intrinsic process in the moss capable of interfering the distribution of elements, then similar uptake patterns will be observed in both types of samples.

2. Material and Methods

2.1. Sampling

Samples of the moss *Pseudoscleropodium purum* (Hedw.) M. Fleisch were collected on at least two occasions between 1999 and 2008 from 33 control sites (Fernández *et al*., 2007) and 43 industrial sites (some of which were sampled on several occasions: Varela et al., submitted) in Galicia (NW Spain) The annual production levels of each of the factories concerned are listed in Table 1. Two sampling sites (SS) were established in the surroundings of each possible source of contamination (see Fernández et al., 2007 and Varela *et al*., submitted for further details).

2.2. Chemical analysis

Details about the chemical analysis of the samples are described elsewhere (Fernández *et al.*, 2007 and Varela *et al.*, submitted). Briefly, prior to the analysis, samples were cleaned to remove adhered material; apical portions (3-4 cm long) were separated from the shoots and the material was homogenized and digested with HNO3 in a microwave oven. The concentrations of Al, Ba, Be, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Sr, V and Zn were determined by flame or graphite furnace atomic absorption spectrometry. Arsenic was determined by atomic fluorescence spectrophotometry, and Hg was determined in an elemental analyzer. Quality controls results (*i.e.* recoveries, quantification limits and reproducibility) were satisfactory (see Varela *et al.*, submitted).

2.3. Statistical analysis

The relationships between the elements measured in the moss samples were analysed by a principal component factorial analysis (PC-FA), according to Mardia *et al.* (1979); varimax rotation was included as a final step to simplify the loading structure. In factorial analysis, the principal component method has the advantage that it does not require the assumption of normally distributed data. The analysis was applied separately to the data from the control sites and from the industrial sites. All analyses were carried out with R statistical software (R Development Core Team 2008).

Table 1. Goods manufactured and annual production (1: t year^{-1}; 2: m^3 year^{-1}) for the factories under study. Source of production data: a: Regional Goverment (Xunta de Galicia, www.xunta.es); b: E-PRTR, c: business website; n.a.: not available.

Industry	Product	Production
01	Chlor-alkali and derived products	$33,552^{1,a}$
02	Bricks	$70,000^{1,a}$
03	Bricks and tiles	$>20,000^{1,b}$
04	Bricks and filler blocks	$>20,000^{1,b}$
05	Bricks and tiles	$>20,000^{1,b}$
06	Ceramics, Tiles, Porcelain	$>20,000^{1,b}$
07	Bricks, Thermo-clay blocks	$29,000^{1,b}$
08	Bricks and tiles	$>20,000^{1,b}$
09	Bricks	$>20,000^{1,b}$
10	Bricks and tiles	$90,000^{1,c}$
11	Tiles	$63,985^{1,b}$
12	Bricks	$>20,000^{1,b}$
13	Bricks	$>20,000^{1,b}$
14	Cannery and cogeneration	$23,000^{1,b}$
15	Cannery and cogeneration	$>54,000^{1,b}$
16	Animal feed and cogeneration	$140,000^{1,b}$
17	Aviculture and cogeneration	$141,000^{1,b}$
18	Cannery and cogeneration	$>20,000^{1,c}$
19	Cannery and cogeneration	$60.000^{1,a}$
20	Electrolytic coatings	$>36,000^{1,c}$
21	Bioethanol	$138,723^{2,b}$
22	Cement and clinker	$369,910^{1,b}$
23	Iron and steel	$750,000^{1,b}$
24	Silicon and manganese	n.d.
25	Silicon and manganese	n.d.
26	Glass, glassfibre	$>6,000^{1,b}$
27	Mineral products	$>134,500^{1,b}$
28	Aluminium, Alumina and derivatives.	n.d.
29	Aluminium profiles for industrial use	$81,500^{1,c}$
30	Aluminium profiles for industrial use	$70,000^{1,c}$
31	Reutilization of oil	$>3,000^{1,b}$
32	Wood boards	$184,032^{2,b}$
33	Wood boards	$184,523^{2,b}$
34	Wood boards	$>5,500^{2,b}$
35	Wood boards	n.d.
36	Wood boards	n.d.
37	Wood boards	$318,873^{2,b}$
38	Wood boards	n.d.
39	Wood boards	$135,000^{1,b}$
40	Wood boards	n.d.
41	Glues	$43,990^{1,b}$
42	Paper and cardboard	$23,315^{1,b}$
43	Urban waste incineration	$541,759^{1,b}$

3. RESULTS

The data were not symmetrically distributed, especially the data from the industrial environments; some of the concentrations were very high, yielding

Table 2. Factor loadings and uniqueness of a Component Factor Analysis carried out with element concentrations in moss samples (*Pseudoscleropodium purum*) collected from background sites and sites surrounding different factories. F_b: factors for background sites; F_i: factors for industrial sites; U: uniqueness value. Values shown in bold type correspond to elements with the highest loadings.

	Background					Industrial				
Metal	F_b1	F_b2	F_b3	F_b4	U	F_i1	F_i2	F_i3	F_i4	U
Al	**-0.49**	0.11	**0.84**	0.08	0.02	-0.05	0.02	-0.11	**-0.72**	0.46
As	-0.04	-0.04	**0.58**	-0.02	0.66	-0.06	0.02	**-0.99**	-0.06	0.02
Ba	-0.08	-0.05	0.00	**-0.62**	0.61	-0.07	0.11	-0.13	**-0.92**	0.12
Be	-0.18	0.00	**0.53**	-0.13	0.67	-0.01	-0.01	**-0.42**	**-0.50**	0.57
Cd	0.10	**0.49**	-0.05	**0.33**	0.64	-0.17	**0.75**	0.02	-0.04	0.41
Co	**-0.56**	0.14	**0.25**	-0.06	0.61	**-0.57**	0.19	-0.32	-0.04	0.53
Cr	**-0.89**	-0.06	**0.30**	0.04	0.11	**-0.91**	-0.05	-0.05	0.04	0.16
Cu	**-0.34**	**0.55**	-0.16	0.05	0.55	**-0.84**	**0.41**	0.06	0.02	0.12
Fe	**-0.66**	0.06	**0.44**	-0.09	0.36	**-0.81**	0.17	-0.23	**-0.34**	0.14
Hg	0.01	**0.57**	0.17	-0.07	0.64	-0.13	**0.90**	0.11	0.02	0.16
Mn	0.01	0.20	-0.01	**-0.51**	0.70	-0.25	0.08	0.03	-0.17	0.90
Ni	**-0.93**	0.10	0.14	-0.07	0.10	**-0.76**	0.25	0.05	0.05	0.35
Pb	-0.17	**0.70**	0.13	-0.21	0.42	-0.14	**0.89**	0.02	-0.02	0.19
Sr	0.13	-0.06	**0.39**	**-0.55**	0.52	-0.07	0.26	-0.14	-0.15	0.88
V	**-0.76**	-0.02	-0.11	0.05	0.41	-0.07	-0.07	**-0.54**	-0.23	0.65
Zn	0.02	**0.38**	-0.09	-0.02	0.85	-0.26	**0.94**	0.00	-0.06	0.04

distributions with long right tails. Such high values can influence the result of the factorial analysis by generating spurious correlations that become associated with a fictitious factor. To test for the presence of such effects and to reduce the influence of the higher values, we applied logarithmic transformations to the concentration values before repeating the analysis. This did not affect the structure of the factor loadings for the background samples (the order of factors 3 and 4 was reversed after the transformation, but the loadings remained stable), and factors 1 and 2 for the data from the industrial sites were also stable. However, factors 3 and 4 for the industrial sites were not stable and collapsed into a single factor with larger loadings for Al, As, Ba, Be, Fe and V when transformed data were used.

The results of the factorial analysis with untransformed data are summarized in Table 2, which includes the loading values and the values indicating the uniqueness of each element. Four factors were selected for the final analysis because previous trials had shown that the first four factors absorbed most of the variance explained by the model. The elements with the highest loadings for each factor are highlighted in the table. We did not use any particular criterion for selecting the elements and simply chose the highest values

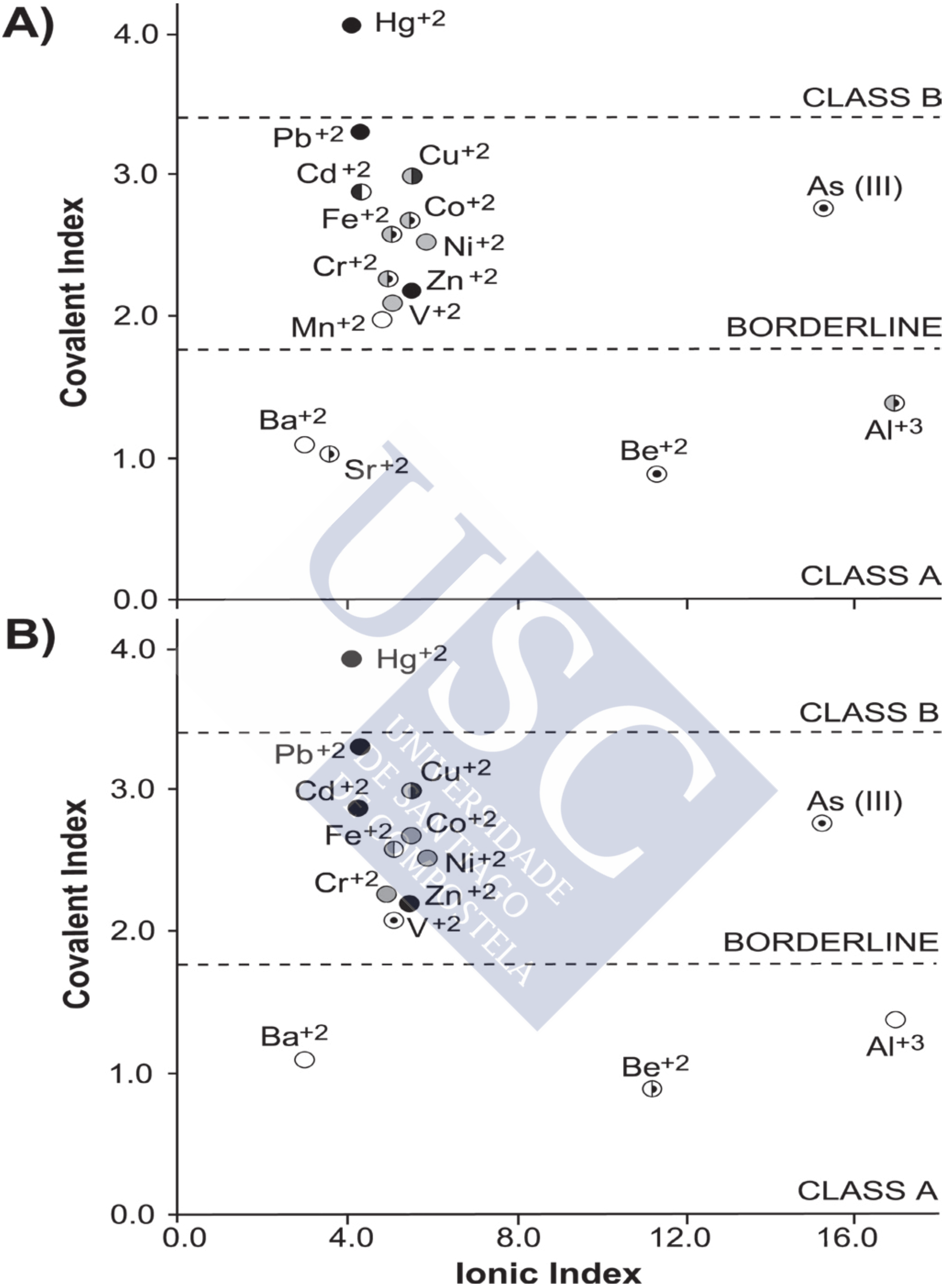

Figure 1. Ionic and covalent binding indices (adapted from Nieboer and Richardson, 1987) of elements included in the corresponding factors of the Component Factor Analysis (grey circles: factor 1; black circles: factor 2; dot inside circles: factor 3; white circles: factor 4) carried out with metal and metalloids determined on the moss samples (*Pseudoscleropodium purum*) collected in backgrounds sites (A) and sites around different factories (B). The covalent binding index is represented by $X^2_m r$ and the ionic binding index by $Z^2 r^{-1}$, where X_m is the metal-ion electronegativity, r is the ionic radius and Z is the formal charge (for details, see Nieboer and Richardson, 1987).

within each factor. Thus, for example, in Fb1, the loading value for Cu was 0.34. Although this may not appear to be very high, it is at least two times higher than the other loading values (< 0.20). We will discuss the real significance of these values in the following sections.

Comparison of the stable factors and their loadings obtained in each analysis (*i.e*. background and industrial data) reveals some interesting similarities. The first factors for the control sites (F_b1) and for the industrial sites (F_i1) are similar as both include Co, Cr, Cu, Fe and Ni, although F_b1 also includes Al and V. Factors F_b2 and F_i2 are also similar because the highest loadings correspond to Cd, Cu, Hg, Pb and Zn.

The uniqueness of each element, as identified by the FA, was generally higher in the samples from the control sites (except for Al, Cr, Mn, Ni, Sr and V) than in samples from the industrial sites. This indicates a higher percentage of variance in the data explained by the FA, with the aforementioned exceptions, for the moss samples from the industrial sites. Thus, the explained variance ranged between 30 and 50% (i.e. U=0.5-0.7; Table 2) for the samples from control sites and between 80 and 100% (i.e. U=0.02-0.2) for samples from the industrial sites.

4. Discussion

4.1 Relationships between factors and the physicochemical properties of elements.

The presence of common factors in both analyses suggests that the origin of the contaminants is not the cause of the relationships between the metals, but rather that the bioconcentration of these elements can be explained by the adsorption processes in moss and physicochemical characteristics of the elements. The groups of elements associated with the factors identified by the FA may be similar to those classified by Nieboer and Richardson (1980) on the basis of cation binding properties involved in organic matter binding. These authors established two indices to quantify the ionic and covalent nature of ions, which also determined the types of organic radicals to which the ions preferably bind. In this classification, the ions are divided into class A, class B and borderline. The values of the ionic and covalent binding indices of the elements included in the different factors analysed in the analyses carried out in the present study are shown in Fig. 1 (adapted from Nieboer and Richardson, 1980). Factors F_b2 and F_i2 (Table 2) include Cd, Cu, Hg, Pb and Zn (Fig. 1) (situated close to each other in the periodic table), which have high and similar ionic binding index values (ca. 5) and also high covalent binding index values (ca. 3.2-4.2). In addition, Cd, Hg and Pb are almost always characterized by significant correlations between the concentrations in bulk deposition and in the moss tissue and usually by high coefficients of determination, whereas this is true for Zn in most cases and for Cu in half of the cases (Aboal *et al*., 2010). The significant correlations may be due to the high covalent binding index values for these elements. Likewise, Cu, Hg and Pb (for which the values of the covalent binding index are highest) tend to occur at higher concentrations in the basal part of the shoots than in the apical part (Brown and Brown, 1990; Leblond *et al*., 2004; Boquete *et al*., in press). The differences along the shoot may be due to the low mobility of these elements, which are strongly bound to the cell wall and membrane (Brown and Brown, 1990; Boquete *et al.*, in press).

Factors F_b1 and Fi1 both include Al, Co, Cr, Cu, Fe, Ni and V. For all of these, except Al, the values of the ionic binding index are between 5 and 6, and the values of the covalence binding index are between 2-2.7; almost all of these elements are also close to each other in row 4 of the periodic table. In other words, the elements included in these factors have very similar characteristics to those in F_b2 and F_i2, but with lower covalent binding index values. Aluminium (which has different binding properties) appears to be less closely associated with factor Fb1 than the other elements. It has previously been observed that the concentrations of Co, Fe and V in the bulk deposition and in the moss tissue are usually significantly correlated, whereas this is only occasionally true for Cu and Ni and very rarely true for Cr (Aboal *et al*., 2010).

Factor F_b3, which includes Al, As, Be, Co, Cr, Fe and Sr, can be interpreted as a grouping of elements with variable covalent binding index values but high ionic binding index values. The ionic binding index values for Co, Cr are Sr are low and therefore these elements would not be included in this definition; however, their loadings in this factor are low and they are more closely associated with other factors (F_b1 and F_b4), which appears to indicate a weak relationship with this axis.
loadings. A low ionic binding index value is a common characteristic of these elements. Manganese might be expected to be included in factor F_b1 because of its low covalent binding index value; however, it has recently be recommended that Mn should not be included in moss biomonitoring studies (Boquete *et al*., 2011) as increased concentrations will only be observed in moss tissues under certain circumstances, and for certain types of emission, associated with increased deposition of the element.

Table 3. Metals and metalloids determined in different moss species collected around different factories (CA: chlor-alkali plant; FS: ferrous-smelter; MI: mineral factory; NF: non ferrous-smelter; PP: power plant). The concentrations of the metals indicated in bold type were significantly correlated with the distance to the focus.

Reference	Factory	Metals
Turkan et al. (1995) F	S	**Cd, Cr, Fe, Mn, Pb, Zn**
Brumelis et al. (1999) F	S	Cu, Pb, Zn
Fernández et al. (2000)	CA	**Hg**
Poykio et al. (2001) F	S and MI	**Cr, Ni, Zn**
Ceburnis et al. (2002) P	P	**Cr, Ni, V**
Zechmeister et al. (2004) F	S	Al, As , **Cd**, Co, Cr, **Cu**, Fe, **Hg**, Ni, V, **Zn**
Schintu et al. (2005) N	F and 2 PP	**Cd**, Cr, Cu, **Pb, V, Zn**
Uyar et al. (2007)	PP	**As, Co**, Cr, Cu, **Fe**, Ni, **Pb**
Suchara et al. (2008) C	A	**Hg**
Gónzalez-Miqueo et al. (2010)	FS	**As**, Cd, **Cr, Cu**, Hg, **Ni, Pb, V**, Zn
Gónzalez-Miqueo et al. (2010)	FS	**As**, Cd, **Cr, Cu**, Hg, **Ni**, Pb, **V**, Zn
Balabanova et al. (2010) M	I	**Al**, As, Ba, **Cd**, Co, Cr, **Cu, Fe**, Mn, Ni, **Pb** ,Sr, **Zn**
Bačeva et al. (2012) F	S	Al, As, Ba, Be, **Cd, Co, Cr**, Cu, **Fe**, Hg, Mn, **Ni, Pb**, Sr, V, **Zn**

Factor F_b4 includes Ba, Mn and Sr, all with moderate was Pseudoscleropodium purum, and as the samples were collected in the same region as in the present study, the findings are highly relevant to this discussion. These authors showed that most of the binding sites could (tentatively) be assigned to carboxyl, phosphodiester and phosphoryl groups (2.46 10-1 mmol g^{-1}), to amine groups (1.02 10-1 mmol g-1) and to polyphenols (2.06 10^{-1} mmol g^{-1}). The first three groups contain oxygen and preferably bind elements with low covalence binding index values (class A elements sensu Nieboer and Richardson, 1980), whereas amine, sulphydryl and other groups without oxygen preferably bind elements with high covalence binding index values (class B). Elements with intermediate covalence binding index values (borderline) can bind to all types of ligands. Thus, factors F_b1 and F_b2 could be interpreted as being produced by the first group of ligands and the amines respectively.

If this hypothesis is correct, the correlations explained by the factors should be due to changes in surface properties of moss, not to differences in the origin of the elements. This may explain the similar structure of the factors in both background and industrial sample sets. Unfortunately, there are no available data on the variability of the surface properties of the mosses to support this supposition.

4.2 Physicochemical properties of the moss surfaces

González and Pokrovsky (2014) reported the first description of the chemical properties of the surface of mosses. One of the four species included in this study was *Pseudoscleropodium purum*, and as the samples were collected in the same region as in the present study, the findings are highly relevant to this discussion. These authors showed that most of the binding sites could (tentatively) be assigned to carboxyl, phosphodiester and phosphoryl groups (2.46 10^{-1} mmol g^{-1}), to amine groups (1.02 10^{-1} mmol g^{-1}) and to polyphenols (2.06 10^{-1} mmol g^{-1}). The first three groups contain oxygen and preferably bind elements with low covalence binding index values (class A elements sensu Nieboer and Richardson, 1980), whereas amine, sulphydryl and other groups without oxygen preferably bind elements with high covalence binding index values (class B). Elements with intermediate covalence binding index values (borderline) can bind to all types of ligands. Thus, factors F_b1 and F_b2 could be interpreted as being produced by the first group of ligands and the amines respectively.

If this hypothesis is correct, the correlations explained by the factors should be due to changes in surface properties of moss, not to differences in the origin of the elements. This may explain the similar structure of the factors in both background and industrial sample sets. Unfortunately, there are no available data on the variability of the surface properties of the mosses to support this supposition.

4.3 Similar studies

Other studies have applied factorial analysis to the concentrations of contaminants in mosses growing in industrial sites (Kuik *et al*., 1995; Balabanova *et al.*, 2010; Bačeva *et al.*, 2012). In the first of these studies, Kuik *et al.* (1995) analysed samples of Pleurozium schreberi collected from different sites in The Netherlands and obtained 8 factors, one of which included Cr, Fe, Ni and V (similar to factor F_b1

in the present study, Table 2) and another included Co, Fe, Hg, Ni, Pb and V (similar to a combination of F_b1 and F_b2 or F_i1 and F_i3). Bačeva et al. (2012), who analysed different species of moss (*Campothecium lutescens*, *Hypnum cupressiforme* and *Homolothecium sericium*) collected in the vicinity of a ferronickel smelter plant in Macedonia, obtained three factors; one of these included As, Cd, Cu, Hg, Pb and Zn (similar to F_b2 or F_i2), and another included Co, Cr, Cu, Fe and Ni (similar to F_b1 or F_i1 in the present study). In both of the aforementioned studies, the authors indicate that these groupings are associated with different sources of contamination (*e.g*. fuel combustion, metallurgy, etc.). However, as already mentioned, the groupings are probably due to the physicochemical characteristics of the elements and their interactions with the functional groups of the moss and/or particles.

On the other hand, in a comparison of the correlations between the concentrations of contaminants in different species of moss in the industrial sites and the distance from the source of emission reported in other studies (Table 3), we found that the elements for which the highest number of correlations are reported are those included in the pattern observed in the present study (*i.e.* Co-Cr-Fe-Ni and Cd-Cu-Hg-Pb-Zn). Significant correlations were observed for these elements in at least 50% of the studies: for Fe in 80% (4 out of 5 studies); for Pb (6/8) and Cd (5/7) in more than 70% of cases; for Cr (6/10) and Ni (5/8) in more than 60%; and finally for Co (2/4), Cu (4/8) and Hg (3/6) in 50% of cases. All of these elements are characterized by being class B or borderline type ions, with high covalent binding index values. Elements such as Cd, Hg and Pb, with high covalent binding index values, are the elements most commonly measured in moss biomonitoring studies in the last decade (*e.g.* Harmens *et al.*, 2010, 2012). Lead was the element for which the technique was first successfully applied (Rühling and Tyler, 1968).

4.4 Other effects related to the adsorption properties of the moss

The physicochemical characteristics of the elements will also influence the concentrations of elements that are taken up by mosses via cationic competition and displacement. In fact, in a study in which the sequential elution technique was used to identify the cellular location of the contaminants (Pérez Llamazares et al., 2011), metals with high covalent binding index values (*e.g*. Co, Ni and Pb) were use to extract metals with lower values of this index (*e.g*. all class A). Likewise, chelating agents with S functional groups were required (e.g. dimercaprol; Pérez Llamazares *et al*., 2010) were used to extract class B elements. The effect of the different affinity of the ions for different functional groups will affect both the adsorption process in the moss and the adsorption of any particles deposited on the moss surface, and both may determine the amounts of each element accumulated.

5. Conclusions

The common pattern of variation in the concentrations of elements in moss samples from both industrial and control sites, which were found to be related to binding properties of the elements, suggest that the physicochemical characteristics play an essential role in the uptake of metals and metalloids from the atmosphere. Depending on whether the elements are class A, B or borderline, the presence of different functional groups on the cell wall will determine uptake/retention by moss. However, as the uniqueness value (Table 2) never equals 0, some of the variability in the concentrations of the elements under study (the amount will depend on the element under consideration) can be attributed to causes other than the inherent physicochemical characteristics of the moss.

From a practical viewpoint, the main consequence of this phenomenon is that by overlooking the adsorption properties of the moss erroneous conclusions could be reached in biomonitoring studies in which multiple correlations among elements are used to determine a common origin for the contaminant.

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CHAPTER 05


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Physiological and Growth Responses of Transplants of the Moss *Pseudoscleropodium purum* to Atmospheric Pollutants

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Abstract Terrestrial mosses are used as biomonitors to assess atmospheric pollution. The aim of this study was to evaluate the effects of chemical contaminants on the physiological responses of mosses. For this purpose, we transplanted clean moss samples to five sampling sites at different distances from a steelworks factory, for different lengths of time (four periods of 1 month, two periods of 2 months and one period of 4 months). At each sampling site, we collected bulk deposition samples, and we also measured the concentrations of Al, As, Cd, Cr, Cu, Fe, Hg, Ni, Pb, V and Zn in the moss tissues and determined the chlorophyll content (CHL) index, Fv/Fm ratio and moss growth rate, with the following aims: (a) to determine the relationships between the concentrations of elements in bulk deposition and moss samples, physiological traits and growth of the mosses; and (b) to determine whether such relationships are affected by the duration of exposure and level of contamination. Although we did not identify any such relationships, the CHL index and growth rate were both lowest at high concentrations of metals and metalloids in the moss tissues, which suggest the existence of a threshold type of response for both variables.

Keywords Bulk precipitation · Chlorophyll fluorescence · Spectral reflectance · Terrestrial mosses · Steelworks

1 Introduction

Biomonitoring is currently one of the most commonly used environmental surveillance tools. The technique involves measuring the concentrations of anthropogenic contaminants in living organisms. It has been known for several decades that anthropogenic activities release numerous chemical compounds into the environment; according to recent data from the European Inventory of Existing Commercial Chemical Substances (EINECS; http://ec.europa.eu/environment/chemicals/exist_subst/einecs.htm), about 100,000 compounds are produced in Europe every year. However, our capacity to analyse and detect many of these compounds, particularly in natural matrices, is very limited (Wagner 1994). Because of the huge number of existing compounds and the associated analytical limitations, it can often be difficult to decide which compounds should be studied and quantified in biomonitoring studies and/or surveys. Moreover, the

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presence of a particular substance in an organism does not necessarily indicate toxic effects, which will depend on other factors including the bioavailability of the contaminants, their location in the organism, their possible transformation and the associated toxicokinetic reactions.

As an alternative to measuring the types and amounts of chemical substances in biomonitors, the effects that are produced in such organisms can be measured directly as changes in morphological and physiological traits. Terrestrial bryophytes are used to biomonitor atmospheric contamination (Onianwa 2001). However, very few studies have determined the effects of the contaminants on physiological traits of moss transplants in the field (Tuba et al. 1997; Aboal et al. 2008), in laboratory experiments (Takács et al. 1997; Shakya et al. 2008; Sun et al. 2009; Tuba et al. 2010) or both (Tremper et al. 2004). Although the problems associated with the selection and analysis of contaminants can be avoided by the use of moss transplants, this approach does not enable identification of the causal agents of any toxic effects, because of the difficulty to determine the entire chemical compounds present in moss samples. The response of moss to contamination will be the result of a sequence of processes associated with atmospheric contaminants: emission → inmission → moss uptake (total load) → moss bioconcentration → physiological alterations → damage responses. Although some of these processes have been studied, especially inmission (measured as bulk precipitation and total burdens in moss, e.g. Ross 1990; Berg et al. 1995; 1997), no studies have considered all of these processes together to date.

We chose the surroundings of a steelworks factory known to emit heavy metals and metalloids (Fernández et al. 2007) as experimental site for exposing transplants of terrestrial mosses for different periods of time to investigate the sequence of processes. We considered the changes in concentrations of elements in moss tissues, chlorophyll fluorescence data and spectral reflectance parameters as measures of physiological alterations, and we considered differences in moss growth as a measure of damage. The specific objectives of the study were as follows: (a) to determine any relationships between the concentrations of elements in bulk deposition and in moss, physiological traits and growth of the moss; and (b) to determine whether any such relationships are affected by the duration of exposure to the contaminants.

2 Material and Methods

2.1 Sampling and Moss Transplant Preparation

The study was carried out at five sampling sites (SS) in Galicia (NW Spain). Four of the SS were located in the surroundings of a steelworks and were placed in different directions at increasing distances from the factory (SS1 to SS4). The remaining (control) SS (SS5) was located in a rural area at a distance of 8,150 m from the steelworks. Samples of *Pseudoscleropodium purum* (Hewd.) M. Fleisch were collected at each of the SS. In the laboratory, segments of the moss were measured with a digital caliper (3.0±0.007 cm, mean±0.95 confidence interval) and then excised from the shoots. The samples were sieved on a plastic mesh (0.7 cm) and washed three times, with shaking for 1 min (at first with distilled water and then with double-distilled water), to remove plant remains, epiphytes, adhered edaphic particles, etc. Finally, aliquots of the material (5-g fresh weight) were weighed for preparing each transplant, as follows.

The moss apices were placed on a capillary mat on top of a plastic container, with the short ends of the mat inserted through slits positioned close to the edge of the lid of the container. The container was filled with distilled water, to maintain a high relative humidity and prevent the moss suffering from hydric stress. The moss, capillary mat and the lid of the container were then covered with plastic netting (mesh size 0.5 cm), to ensure that the moss remained in contact with the water and also to prevent it from being blown away by the wind. In each SS, three such transplants were placed inside a plastic container, which was covered with a plastic shading net to reduce the effect of direct solar radiation and to provide further protection against the wind.

The transplants were exposed in situ, for four periods of 1 month (designated 30-I, 30-II, 30-III and 30-IV), for two periods of 2 months (60-I and 60-II) and for one period of 4 months (120). After each transplant was removed, it was replaced with a new transplant. The study was carried out between 14 September 2010 and 17 January 2011.

2.2 Bulk Deposition

Bulk deposition samples were collected for the 4-month study period, by placing three collectors in each of the five SS. The collectors consisted of plastic funnels connected by plastic hosing to a plastic container. The funnels were placed at a height of 2 m above the ground. Every month, the volume of the water in each collector was measured, and aliquot was removed and acidified with hyperpure 1 % HNO_3, for posterior analysis. The concentrations of elements in the water samples were determined by ICP-MS (Varian 820-MS). The concentrations of Hg were below the limit of quantification (<0.05 pg g^{-1}). The recovery rates of the elements in the reference materials ranged between 68 % for Al and 123 % for Cu.

2.3 Physiological and Growth Measurements

2.3.1 Chlorophyll Fluorescence

Chlorophyll fluorescence was measured by the saturation pulse method (Schreiber et al. 1998), in which measuring light and saturating light pulses (>4,000 μmol photons m^{-2} s^{-1}, 0.8 s of pulse length, actinic white light) are applied through a fibre-optic cable at a 60° angle relative to the sample and at a 12-mm distance from the sample, with a portable fluorometer (MINI-PAM photosynthesis yield analyser; Walz GmbH). Measurements were made on ten apical segments per sample. The chlorophyll fluorescence parameter recorded was the maximum quantum yield of photosystem II (Fv/Fm), which estimates the efficiency of excitation energy captured by open PSII reaction centres (Butler and Kitajima 1975). The Fv/Fm ratio was determined as (Fm-F_0)/Fm (Bolhàr-Nordenkampf et al. 1989), where F_0 and Fm are, respectively, the minimal and maximal fluorescence yields of a dark-adapted sample, with all PSII reaction centres fully open (i.e. all primary acceptors oxidised). This parameter was measured after a 30-min period of dark adaptation, which is considered to be a sufficient time to allow all PSII reaction centres to open (Roiloa and Retuerto 2005). The Fv/Fm data for November (period 30-II) were lost, and therefore there are no data available for the first bimonthly period (60-I).

2.3.2 Moss Spectral Reflectance

Immediately after measuring the chlorophyll fluorescence, the spectral reflectance parameters were determined (at wavelengths between 300 and 1100 nm), in the same apical segments, with a portable spectrometer (UniSpec Spectral Analysis System, PP Systems). The reflectance spectra were calculated by dividing the spectral radiance of the moss by the radiance of a reflective white standard (Spectralon Reflectance Standard, Labsphere). The spectral reflectance parameter considered was the chlorophyll content (CHL) index, which was calculated as R_{750}/R_{700}, where R_{750} and R_{700} are reflectance at 750 and 700 nm, respectively. The CHL index is correlated with the chlorophyll content of leaves (Wood et al. 1993; Lichtenthaler et al. 1996).

2.3.3 Growth of Moss Transplants

In the laboratory, the length of the apices was measured with a digital caliper. To determine the growth rate of the moss transplants during the exposure period, the mean length of the apices prior to exposure was subtracted from the final mean length of the apices.

2.4 Metal Content in Mosses

After measuring the physiological and growth parameters, the samples were homogenised in an ultracentrifugal mill (Retsch ZM200, metal free), and HNO_3 (65 % analytical grade) was added to digest the samples (in a microwave oven). The concentrations of Al, Cd, Cr, Cu, Fe, Ni, Pb and Zn were determined by flame atomic absorption spectrometry (Perkin Elmer 2100); when the values were below the limits of quantification of this method, graphite chamber atomic adsorption spectrometry (Perkin Elmer AAnalyst 600) was used to determine the concentrations. The concentrations of Hg were determined in an element analyser (Milestone DMA80). The rates of recovery of the elements in the reference material ranged between 77 % for Hg and V and 121 % for Pb.

2.5 Statistical Analyses

Kolmogorov–Smirnov and Levene tests were used to check the normality and homoscedasticity of the data (i.e. requisites for parametric tests), respectively, and when necessary, the data were transformed to meet the requirements of ANOVA tests. When the ANOVA indicated significant differences, the Tukey test was used to establish homogeneous groups. As the transformations used for the growth data were unable to satisfy the requirements for ANOVA, the Scheirer–

Ray–Hare test (a non-parametric test) was used to analyse this data, and a non-parametric post hoc test (Zar 1984) was used to establish homogeneous subgroups of this data.

Spearman's rho test was used to analyse the correlations between the variables under study: bulk deposition–moss concentration (for each element), CHL–moss concentrations, Fv/Fm–moss concentrations, CHL–moss growth and Fv/Fm–moss growth.

All statistical tests were performed with IBM SPSS 20.0.

3 Results

No correlations were found between the concentrations of elements in bulk deposition and in moss, physiological traits and growth of moss.

3.1 Concentration of Elements in Bulk Deposition and Moss Samples

The concentrations of elements in the monthly bulk deposition samples were lower in the control site (SS5) than in the other sites, for most elements and periods, i.e. Al, As, Cd, Fe, Pb, V and Zn in four of the periods; Cu and Ni in three of the periods; and Cr in two of the periods (Fig. 1). This was also true for the concentrations of most elements in the moss. Thus, the concentrations of Cu, Fe, Ni and Pb were lowest in samples from the control site for all four of the monthly periods, and the concentrations of Al, Cr, Hg and Zn were lowest at the control site in three of the monthly periods (Fig. 1). However, the highest concentrations in the bulk deposition did not coincide with those in moss samples. For the bulk deposition, the concentrations of elements (e.g. Al, Cd, Pb) were generally highest in SS4: the concentrations of As, Cd, Cu and Pb were particularly high during 30-I and those of Zn, during 30-III (Fig. 1). In SS1, the concentrations of Cr and Ni in the bulk deposition were also higher during the periods of 30-II and 30-III (Fig. 1). However, in the moss tissue, the highest concentrations were generally restricted to the SS closest to the factory (SS1). The concentrations of elements in the moss transplants also decreased with increasing distance between the sampling site and the steelworks (Fig. 1), and they reached lower levels than the initial concentrations in the moss (e.g. Cr in 30-III and 30-IV).

For the two bimonthly and the one 4-month period, the pattern of concentrations of elements in the bulk deposition was similar to that observed in the monthly periods; the concentrations were lowest in SS5 and highest in SS4 (Fig. 2). Again, the concentrations of Cr and Ni were higher in SS1. The concentrations of all elements, except Hg and V, were highest in SS1. For most of the elements and sampling sites, the concentrations in the mosses increased with the exposure time (Figs. 1 and 2).

These patterns explain why the concentrations of contaminants in the bulk deposition and the transplanted moss samples were not significantly correlated ($p<0.01$) in the monthly, bimonthly or four-monthly periods.

3.2 Concentration of Elements in Moss in Relation to Physiological Traits

The physiological data measured in the moss samples during the monthly, bimonthly and four-monthly periods are shown in Fig. 3. The CHL index only differed significantly ($p<0.05$) between the SS for the moss samples transplanted during the 30-I period; the values of the index were lowest in SS1 and differed significantly from the values in SS5 (control) in this period. Indeed, the value of the CHL index for SS1 in this period was the lowest observed. The moss from SS1 showed clear signs of damage, e.g. the plants were fragile and dark brown in colour (see supplementary data). The value of the CHL index for SS1 coincided with the maximum concentrations of As, Cd, Cr, Cu, Fe, Pb and Zn, and very high concentrations of Al and V, in the moss tissues (Fig. 2). There were no significant differences in the CHL index between the SS for the bimonthly (60-I and 60-II) and four-monthly (120) periods.

The potential quantum yield of PSII (Fv/Fm) differed significantly between SS in the 30-I period and also in the 30-IV period. For the first period (as with the CHL data), the values were lowest at SS1 and significantly different from the control. For the period 30-IV, the values were lowest at SS4, and these were also significantly different from the control values. However, the concentrations of the metals did not increase in the moss tissues (Fig. 1). The Fv/Fm data, which are only available for one bimonthly period, revealed significant differences between the SS; the values were lowest for SS1 and differed significantly

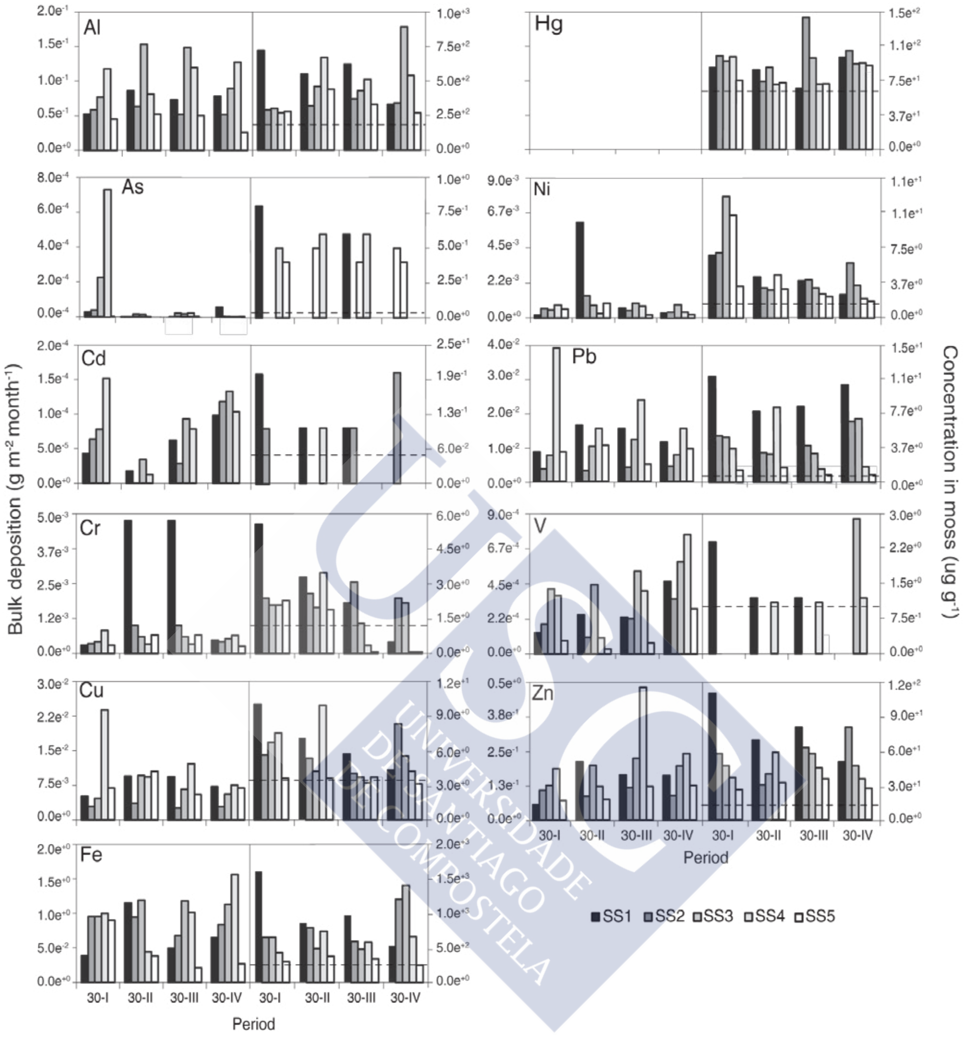

Fig. 1 Concentrations of *Al*, *As*, *Cd*, *Cu*, *Cr*, *Fe*, *Hg*, *Ni*, *Pb*, *V* and *Zn* in the bulk deposition (in gram per square metre) and in moss tissues (in microgram per gram) in each 30-day period: 30-I, 30-II, 30-III and 30-IV. The *horizontal dashed line* represents the concentrations at time zero. When no data are shown, the concentrations were below the limit of quantification

from the values for the other sites (Fig. 3), whereas in the four-monthly period, the only site that differed significantly from the control was SS2.

The monthly concentrations of Cd in moss and the CHL values were significantly correlated ($\rho = -0.55$; $p < 0.01$). However, the concentrations of other elements for other exposure periods were not significantly correlated with CHL or Fv/Fm.

3.3 Physiological Traits and Moss Growth

The growth of the moss varied greatly between SS and exposure periods (Fig. 3). In period 30-I, the growth rate of the moss was significantly lower in SS1 than in SS5, and it was the lowest observed throughout the study. As already mentioned, this low growth rate coincided with the period of greatest accumulation of

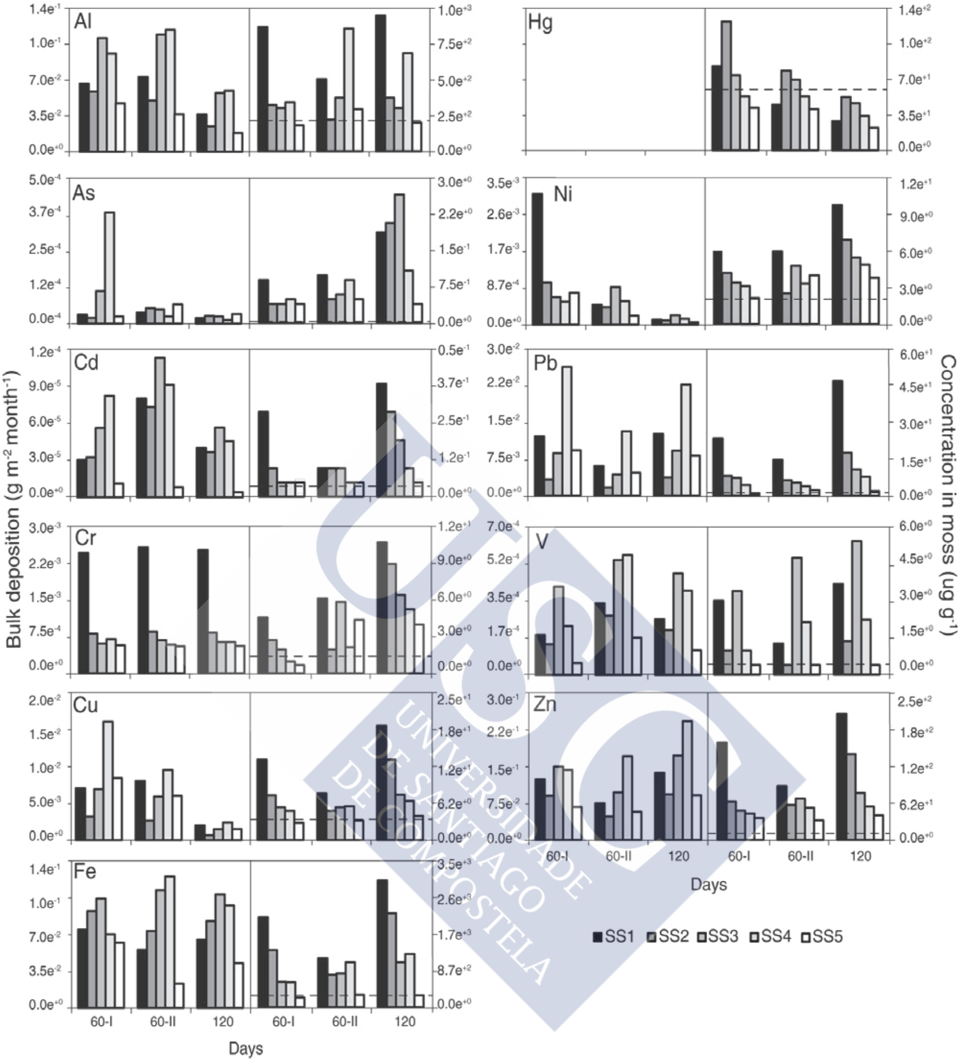

Fig. 2 Concentrations of *Al*, *As*, *Cd*, *Cu*, *Cr*, *Fe*, *Hg*, *Ni*, *Pb*, *V* and *Zn* in the bulk deposition (in gram per square metre) and in mosses tissues (in microgram per gram) during the exposure periods (60-I, 60-II and 120 days). The *horizontal dashed line* represents the concentrations at time zero. When no data are shown, the concentrations were below the limit of quantification

metals in the moss. For the other monthly exposure periods, when differences were observed, the growth rates were lowest in the controls. For the bimonthly periods, growth was only significantly lower than in the control site in the second bimonthly periods in the transplants in SS1 and SS2, and it was similar to the growth observed in the four-monthly period. Decreased growth rates were only observed during long exposure periods. The growth rate of the mosses was 15.8 % after 30 days, but decreased to 5.9 % after 120 days.

The values of the CHL and Fv/Fm indices were not significantly correlated with growth in the monthly, bimonthly or four-monthly periods.

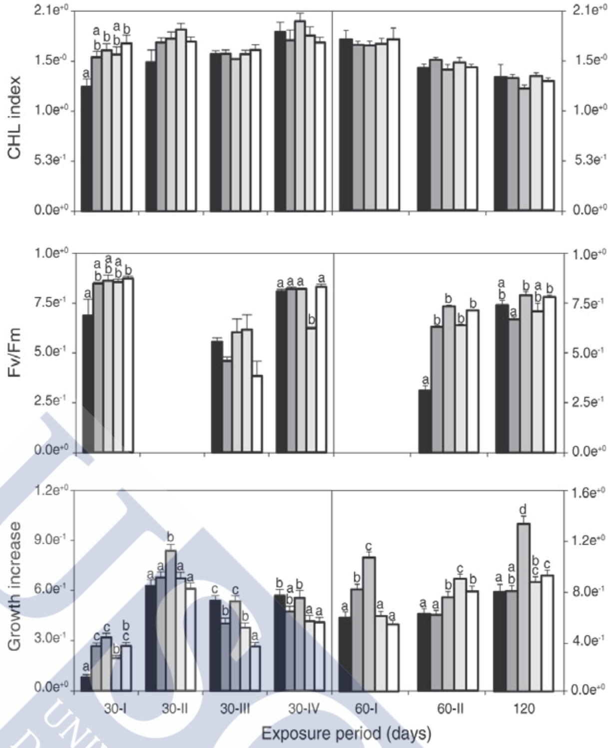

Fig. 3 Mean values (±SE) of chlorophyll content index (*CHL index*), maximum quantum yield of photosystem II (*Fv*/*Fm*) and growth (in centimetre) of mosses during the experimental time (30-I, 30-II, 30-III, 30-IV, 60-I, 60-II and 120 days). Means indicated by *different letters* are significantly different

4 Discussion

Information about the emission of contaminants by the steelworks where the moss samples were transplanted is very scarce. According to The European Pollutant Release and Transfer Register, the factory emitted 214 kg of Cu, 1,890 kg of Pb and 961 kg of Zn per year during the period of 2004–2011 (E-PRTR Spain). However, the factory may also emit other contaminants (not mentioned in the above document). The emissions were evident in the bulk deposition data (Fig. 1), which shows much higher concentrations of these and other elements than at the control site. However, the concentrations of elements in the bulk deposition were not correlated with those in the moss samples. This apparently contradictory result is consistent with the conclusions reached by Aboal et al. (2010), who also reported a lack of any correlation between the concentrations of most heavy metals in bulk deposition and in native moss, except for Cd and Pb. These authors included the following as possible explanations for the lack of correlations: (a) types of inputs (atmospheric, edaphic or plant inputs); (b) the physicochemical characteristics of pollutants and the physicochemical processes in mosses; and (c) biological processes in moss.

In the period 30-I, the concentrations of metals were highest in the moss samples transplanted to the site closest to the steelworks (SS1). Relative to the initial concentrations, the concentrations of Al, Cd, Cu, Cr, Hg, Ni and V increased by two to five times; those of As, Fe and Zn increased by five to ten times; and those of Pb increased by up to 12 times (i.e. to 13 μg g^{-1}). The concentration of chlorophyll, estimated via the CHL index, was lowest (i.e. 1.25) in this period in SS1, which was also the only site in which the concentration was significantly different from the concentration in the control site (Fig. 3). We have only found three previous studies that related the chlorophyll contents to the concentrations of contaminants in moss transplants and that also show different results depending on the contaminant considered. Aboal et al. (2008) used *P. purum* as a biomonitor in the surroundings of an aluminium smelter and observed a reduction in the chlorophyll content (Chl a + b) in relation to the increased tissue loads of F, which reached extremely high levels. However, Bignal et al. (2008), who used *Hylocomium splendens*, *Pleurozium schreberi* and *Racomitrium lanuginosum* to biomonitor an area close to a road with heavy traffic, and Tremper et al. (2004), who used *Rhytidiadelphus squarrosus* and *P. schreberi* exposed in a semi-urban zone and beside a road, did not observe any decrease in the chlorophyll concentration. The latter authors suggested that other factors such as climatic factors and/or interactions with other contaminants would probably have a greater influence on the chlorophyll concentration.

However, in laboratory experiments, reductions in chlorophyll contents have been observed in response to accumulation of metals such as Cd and Pb in *Tortula ruralis* (Takács et al. 1997); Pb and Cr in *Taxithelium nepalense* (Choudhury and Panda 2005); Cu in *R. squarrosus* and *P. schreberi* (Tremper et al. 2004); Cu, Pb and Zn in *Thuidium delicatulum* and *Thuidium sparsifolium* (Shakya et al. 2008); Pb and Ni in *Hypnum plumaeforme* (Sun et al. 2009). However, Tremper et al. (2004) did not observe any effect on the concentrations of chlorophyll in relation to accumulation of Pb and Zn.

Although the results of field studies are not comparable, the results of the laboratory experiments have shown reduction in chlorophyll levels due to metal uptake. The differences are probably due to the fact that higher concentrations of metals were used in the laboratory experiments than those found in the field. Thus, Sun et al. (2009) observed effects with Ni at a concentration of 4 mg and with per Pb at 25 mg, whereas Tremper et al. (2004) observed effects with Cu at a concentration of 286 μg, but did not observe such effects with concentrations of Pb higher than 300 $\mu g\ g^{-1}$ or concentrations of Zn close to 2,000 $\mu g\ g^{-1}$. These results must also be interpreted with care as the metals are usually tested separately in laboratory experiments, and the way in which the metal is administered (i.e. immersion in metal solutions) varies the rate of uptake and toxicity.

The results obtained in the 30-I period may have varied for several different reasons. First, the concentrations of some of the contaminants (metals or metalloids) may have surpassed a level above which effects occur, even though the concentrations were much lower than those that caused a decrease in chlorophyll levels in laboratory experiments (Tremper et al. 2004; Sun et al. 2009). In this type of response, degradation of chlorophylls would only occur in response to accumulation of a contaminant above a certain concentration characteristic for each compound, environment and species. Thus, the level of chlorophyll will not decrease until the threshold concentration of metal is reached. This explains the lack of correlation between the concentrations of metal in moss and the CHL. This type of response may even have occurred in the case of the significant correlation found for Cd. Second, the mixture of contaminants may have exerted a synergistic effect, as demonstrated for Ni and Pb by Sun et al. (2009). Finally, the responses may have varied due to the presence of contaminants other than those detected in the moss and bulk precipitation (e.g. other heavy metals or metalloids, PAHs, etc.), which may act individually or synergistically.

The lowest value of Fv/Fm for the period 30-I was again observed in SS1; however, unlike for CHL, this was not the lowest value in the monthly periods (Fig. 3). The variation in Fv/Fm between monthly periods was greater than for the CHL index. We only found two studies that related the Fv/Fm to the accumulation of contaminants in moss (Tuba et al. 2010; Takács et al. 1997). After carrying out transplants with seven species of bryophytes (the species used in the present study was not included), Tuba et al. (2010) demonstrated that exposure to Pb yielded a reduction in Fv/Fm, in both field and laboratory experiments, with a high level of interspecific variability. The values determined in the present study are within the range of variation found by these authors. Takács et al. (1997) observed decreases in Fv/Fm of 32 and 71 % after exposing *T. ruralis* to different concentrations of Cd and Pb, respectively.

As found for the CHL index, the lowest growth rate observed throughout the study was in SS1 in the period 30-I; the growth rates during this period were the lowest of all the SS and all periods studied. Again, this site was the only one that was different significantly from the control site. We only found one study that investigated the relationship between contamination and growth (Bignal et al. 2008). The authors of this study transplanted specimens of *H. splendens*, *P. schreberi* and *R. lanuginosum* close to a busy road, and they found that the transplants closest to the road grew fastest. They speculated that this may have been due to a fertilisation effect (particularly involving N).

As already indicated for chlorophylls, there may also be a threshold concentration of metals of contaminants above which growth of the moss decreases, as in the period 30-I. There was no correlation between growth and the CHL index, although the minimum values of both occurred during this period. Unlike the CHL index, there was a high degree of variability in growth between SS and exposure periods. Moreover, in the other monthly periods, when growth of moss in SS1 was significantly different from that in the control site, the growth was higher, possibly because of a fertilisation effect similar to that proposed by Bignal et al. (2008).

The damage responses observed in the moss physiology in the period 30-I may be related with the location of pollutants in the different cell compartments (i.e. intercellular, extracellular, intracellular and/or particulate) of moss tissues and their effects (Brown 1995). It have been shown that soluble metals present inside the cells have a potentially immediate effect on the metabolism of the organism and are therefore more likely to cause toxic effects than the extracellular fractions (Brown and Sidhu 1992; Branquinho et al. 1997). However, the extracellular fraction can also provide information about potentially toxic elements that they may pass to the interior of the cell and may even be more harmful than those associated with intracellular particles (Perez-Llamazares et al. 2011). In the case of this study, most of metals are bound to particles deposited on the surface of the moss, so the damage observed in the period of 30-I could be caused directly due to the high extracellular concentration of metals or because the metals bound to particles deposited on the moss surface have been solubilised more rapidly than in the other periods studied.

Longer exposure times did not lead to significant correlations between variables. The effect of longer times is evident in the reduction in the rate of metal uptake in the moss (Figs. 1 and 2) as usually observed in transplanted moss samples (Ares et al. 2012). The chlorophyll content also decreased over time (Fig. 3). In the period 30-I in SS1, the low values of the CHL index and growth recovered on extending the exposure time for another 30 days and reached the same levels as in the control site.

5 Conclusions

The concentrations of elements in bulk deposition and moss samples did not appear to be correlated to physiological traits and growth of moss. Despite the lack of significant correlations, the CHL index and growth were both lower at high tissue concentrations of metals and metalloids. This suggests the existence of a threshold type of response in which the effect (measured with both variables) can only be detected above a certain level of one of these elements and/or of others not determined and/or synergic effects between various elements. Such a response would explain the results obtained in the present study and previous findings. However, this type of response cannot be used to detect concentrations of contaminants below the threshold level. The threshold values must be determined for the CHL index and growth. Among the physiological variables studied, the CHL index appeared to be most useful for biomonitoring purposes as it was subject to the least temporal variability and variability due to environmental differences unrelated to the contamination process. Although the growth rates were more variable, the lowest values coincided with the lowest vales of the CHL index.

The duration of the exposure did not alter the findings as regard the lack of relationships between the processes in the sequence studied. Finally, we recommend that the moss transplants are not exposed for more than 30 days, as there is some recovery in the moss physiology after this time.

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CHAPTER 06


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Establishing Reference Values of Spectral Reflectance Indices in Transplants of *Pseudoscleropodium purum* for Potential Use in Atmospheric Biomonitoring

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Abstract We studied the spatiotemporal variation of the photochemical reflectance index (PRI) and the chlorophyll content index (CHL index) in a terrestrial moss, using self-watering transplants distributed in 8 sampling sites that were sampled periodically during up to 4 years. We did not detect any seasonal patterns or difference between the studied sites, and therefore the variation across the sites reflected the influence of environmental variables in the area. We established the reference thresholds associated with physiological stress as −0.212 for PRI and 1.195 for CHL index. Our findings could be applied for biomonitoring atmospheric contamination on the basis of the physiological stress shown by the moss used.

Keywords Photochemical reflectance index · Chlorophyll content index · Correlograms · Terrestrial moss

Native and transplanted terrestrial mosses are commonly used as biomonitors of atmospheric contamination (Onianwa 2001). The concentrations of various contaminants in the moss tissues are often used to estimate air quality. However, it may be difficult to decide which contaminants should be monitored when, for example, the source of contamination is unknown. One solution to this problem is to determine the air quality by measuring how it affects physiological traits (e.g. photosynthetic pigment content) in transplanted mosses (Tuba et al. 1997; Tremper et al. 2004; Aboal et al. 2008). Reference (threshold) values, representing unstressed moss samples, are required to enable interpretation of any observed effects. Aboal et al. (2008) reported threshold values for the Chlorophyll *a*/*b* and D665/D665a indices, determined in self-watered transplants of *Pseudoscleropodium purum*. To calculate the reference values, these authors used data from 7 sampling sites that were sampled once every 2 months over a period of 5 years.

Measurement of spectral reflectance parameters has become recognised as a good alternative for chlorophyll estimation to techniques such as liquid chromatography and spectrometry (e.g. Gamon et al. 1997). The main advantages of this technique over traditionally used methods are that it is (1) non destructive, (2) less expensive, (3) faster, and (4) highly replicable. To date, the technique has mainly been used in higher plants. Use of the technique with moss species has been limited (Lovelock and Robinson 2002; Van Gaalen et al. 2007), and it has not been applied in atmospheric biomonitoring studies with mosses.

Therefore, to enable biomonitoring of air quality based on physiological effects in the moss *P. purum*, reference thresholds must be established for the spectral reflectance indices. The objectives of the present study were as follows: (1) to study the existence of seasonal patterns in the chlorophyll content index and the photochemical reflectance index; (2) to study how both indices vary spatially at a regional scale, and (3) to establish reference thresholds of the indices showing physiological stress.

Materials and Methods

Samples of the terrestrial moss *P. purum* (Hewd.) M. Fleisch were collected from a rural area (A Estrada;

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X = 540,947, Y = 472,874; coordinates UTM29N ED50), at a distance of at least 300 m from main roads and centres of population and at least 100 m from other types of roads and isolated houses. Once in the laboratory, the samples were cleaned and 3 cm-long apical segments were separated from the shoots for analysis. Apical tissue was used because it has been demonstrated that young tissue is physiologically the most active part of the plant (Bates 1979). The segments were then washed with distilled water (with shaking) to remove plant remains and minimize the amount of edaphic particles adhered to the surface; aliquots of the tissue (5 g fresh weight) were then prepared for transplanting. For each sample, a self-watering system was prepared by fitting a piece of capillary matting inside the lid of a plastic jar. The ends of the matting were placed so that they protruded through two slots at opposite edges of the lid. The jar was filled with distilled water and the lid was placed on top so that the ends of the capillary matting were in contact with the water. The prepared apical samples were then placed on top of the capillary matting. This system maintained the moss samples damp, thus preventing them from suffering hydric stress. A piece of plastic mesh was placed over the lid of each jar to prevent loss of material. In the field, the transplants were placed inside a stainless steel frame (60 × 60 × 70 cm), which was completely covered with a plastic shading net to reduce the effect of direct solar radiation and to provide protection against the wind (for details, see Couto et al. 2003; Aboal et al. 2008; Fernández et al. 2010).

The study was carried out at 8 sampling sites (SS) in rural areas in northern Galicia (NW Spain) (Table 1). The average distance between SS was 64 km (maximum distance, 120 km). Three replicated transplants were situated in each SS and were left for periods of 56 days at different times between 10 February 2004 and 15 January 2008 (Table 1).

At the end of each 56-day exposure period, the moss transplants were transferred to the laboratory, and a new transplant was exposed. Once in the laboratory, they were washed with double distilled water for 30 s, with shaking, and blotted on filter paper to remove excess water.

The chlorophyll content index (CHL index) and the photochemical reflectance index (PRI) were determined in the apical segments by use of a portable spectrometer (UniSpec Spectral Analysis System, PP Systems). The reflectance measurements required for each index were made at wavelengths between 300 and 1,100 nm and were repeated 6 times in each transplant. The reflectance values were calculated by dividing the spectral radiance of the shoot by the radiance of a reflective white standard (Spectralon Reflectance Standard, Labsphere). The CHL index was calculated as R_{750}/R_{700}, where R_{750} and R_{700} represent the reflectance measurements at 750 and at 700 nm (Lichtenthaler et al. 1996). The values of the CHL index are correlated with the chlorophyll content of leaves (Lichtenthaler et al. 1996). The PRI was calculated as $(R_{539} - R_{570})/(R_{539} + R_{570})$, where R_{539} and R_{570} represent the reflectance values at 539 and at 570 nm (Filella et al. 1996).

Table 1 Codes, UTM coordinates (29T N ED50), altitude of the sampling sites (SS) and sampling periods and number of samples

Code	X	Y	m.a.s.l.	Sampling periods	n
SS1	602,098	4,820,985	540	10/02/04–15/01/08[ab]	23
SS2	582,017	4,806,552	480	10/02/04–15/01/08[ab]	23
SS3	541,107	4,771,581	270	10/02/04–15/01/08[ab]	21
SS4	552,292	4,779,549	440	10/02/04–15/01/08[ab]	23
SS5	583,843	4,837,331	415	14/03/06–15/01/08[b]	12
SS6	606,332	4,721,281	506	29/08/06–15/01/08[b]	9
SS7	606,041	4,796,623	465	15/03/05–30/08/05	4
SS8	565,583	4,781,008	380	15/03/05–30/08/05	4

m.a.s.l Meters above sea level

[a] No data were obtained between 30/08/05 and 14/03/06

[b] No data were obtained between 25/09/07 and 15/01/08

The Shapiro–Wilk test was used to test the normality of each data set prior to statistical analysis. Moreover, the Levene test has verified that there is homogeneity of variances. Time series of data from the four SS in which n > 20 (SS 1-4: Table 1) were analyzed with the autocorrelation function (ACF) and the autocorrelations were graphically represented on correlograms. Correlograms provide an analytical view of the basic structure of a temporal series. This representation allows us to determine if there exists cyclic variation in the behaviour of the temporal series, in our case, any seasonal trends in the reflectance indices.

A paired sample *t* test was then used to test for any differences in the reflectance indices between SS. Pearson's correlation was used to test for any correlations between the values of the reflectance indices obtained for the different SS. These statistical tests were performed with IBM SPSS 20.0. Kernel smoothing was used to estimate the density distribution of the CHL and PRI data, with the Kern-Smooth software package (Ripley 2002) and R software (R Development Core Team 2008).

Results and Discussion

The mean value (±95 % confidence interval) of the CHL index (calculated from the 18 measurements for each SS in each exposure period) varied between 1.63 ± 0.08 (SS8) and 1.31 ± 0.08 (SS6). The coefficient of variation (CV) for each SS was low and varied between 4.9 % (SS8) and

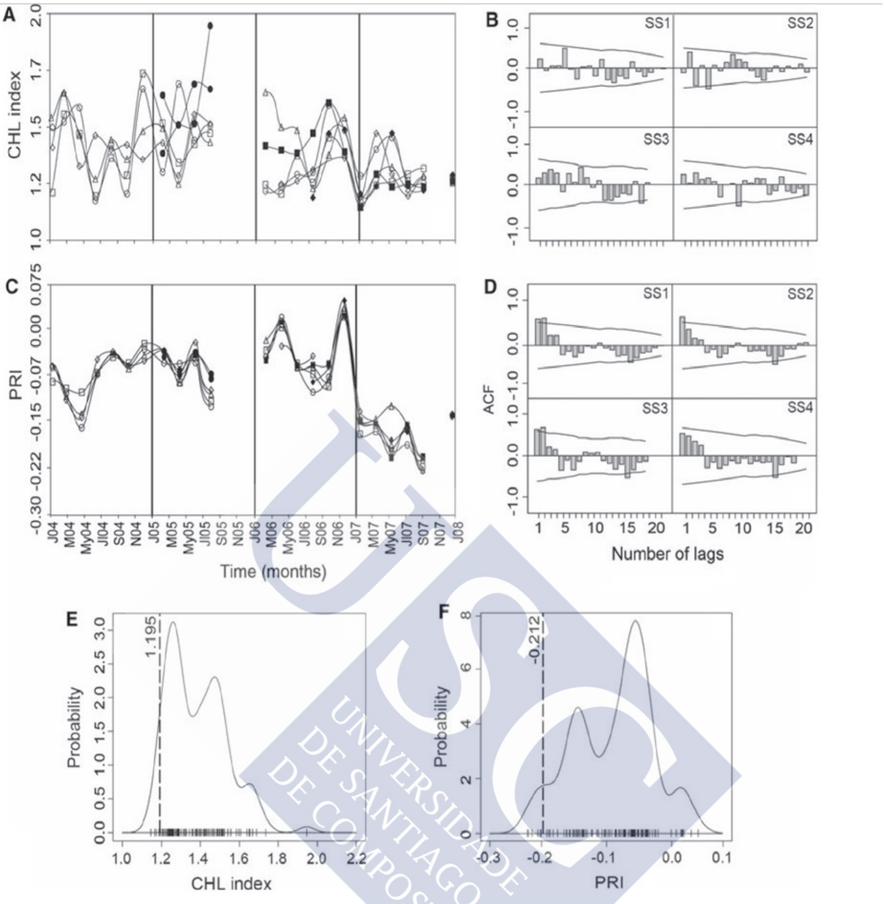

Fig. 1 Time series for **a** the chlorophyll content index (CHL index) and **c** the photochemical reflectance index (PRI) in transplants of *P. purum*, between January 2004 and January 2008. *Open square*: SS1; *open circle*: SS2; *open diamond*: SS3; *open triangle*: SS4; *filled square*: SS5; *filled diamond*: SS6; *filled circle*: SS7 and SS8 (standard errors are omitted for an easy display). Correlograms for **b** the CHL index and **d** the PRI in SS1, 2, 3 and 4. The *vertical bars* represent the coefficients of autocorrelation, and the solid lines the confident interval ($p < 0.05$). Kernel smoothing distribution of **e** the CHL index and **f** the PRI for all available data. *Vertical dashed lines* correspond to the 5 % percentile of the distributions. *Vertical lines* on the secondary *x*-axis represent the position of each value used to construct the distributions

15.7 % (SS7). The mean values of the mean PRI index varied between −0.05 ± 0.02 (SS7) and −0.99 ± 0.05 (SS5). The CVs of the PRI were higher than 40 % for all SS and reached as high as 77.8 % for SS1.

It has been shown that in mosses, the PRI is associated with the xanthophyll cycle, which is involved in the dissipation of the light that is absorbed but not used in photosynthesis, and is therefore related to photosynthetic efficiency (Lovelock and Robinson 2002; Van Gaalen et al. 2007). The CHL index, on the other hand, provides an accurate estimate of the chlorophyll content of the leaves (Lichtenthaler et al. 1996). In the absence of contamination, both indices are affected by different environmental variables (e.g. temperature, moisture, photoperiod and radiation); however, the use of shaded, self-watered transplants eliminates both the hydric stress and any stress arising from exposure to solar radiation. The PRI values in the present study (−0.24 to 0.06) are similar to those reported by Lovelock and Robinson (2002) in Antarctic species such as *Grimmia antarctici*, *Ceratodon purpureus* and *Bryum pseudotriquetrum*, which varied between −0.30 and 0.0. In both the latter and the present study, the variation was higher than that found by Van Gaalen et al. (2007) for *Sphagnum* in North Canada, which ranged between −0.09 and −0.05. The PRI values used in both of these studies was calculated as $(R_{531} - R_{570})/(R_{531} + R_{570})$, as suggested by Gamon et al. (1997).

The time series of CHL index for each SS are shown in Fig. 1a. The values ranged between a minimum of 1.17 (SS1 and 2) and a maximum of 1.95 (SS7). The temporal variation in the data was similar in some SS, and significant correlations were observed between SS1 and SS2 ($p < 0.001$), SS1 and SS4 ($p < 0.05$), and SS3 and SS4 ($p < 0.01$). However, the correlograms (Fig. 1b) did not reveal any seasonal pattern at the scale used (56-day periods) for any of the SS. The time series of the PRI values are shown in Fig. 1c; the values varied more widely than those of the CHL index, from a minimum of −0.24 (SS2 and SS3) to a maximum of 0.06 (SS6). The temporal variation was very similar across all SS, with significant correlations between sites ($p < 0.001$). Again, the correlograms (Fig. 1d) did not reveal any seasonal patterns.

Once the absence of seasonal patterns in all data series was confirmed, we searched for significant differences between SS with respect to any of the indices used. As no significant differences ($p < 0.05$) were found, the values of each index for each of the SS were grouped into a single data set, which was used to estimate density functions (Fig. 1e, f, for the CHL index and the PRI, respectively). Each distribution has two main modes. Finally, the 5 % percentile value is indicated in each distribution.

The facts that there were no seasonal patterns in either CHL or PRI and that the values of the indices did not differ between SS, and were even correlated between some SS, indicated the existence of a natural variability caused by the environmental conditions that affect the entire area. The study area includes two large electricity generating stations, which may act as large scale sources of contamination. However, if the contamination affected the indices in a particular period or SS, lower values different from those corresponding to SS6, which is part of The European Monitoring and Evaluation Programme (EMEP), should have been obtained.

The variability in the combined data set (comprising the data from the different SS and for the different exposure periods) reflects the natural variability in moss transplants not subjected to the physiological stress caused by atmospheric contamination. The distribution of these data can be used as a reference distribution, thus enabling the damage threshold to be established (as the 5 % percentile of the distribution).

In conclusion, no seasonal patterns were observed in the values of the spectral reflectance indices (CHL index and PRI) in shaded, self-watered transplants of *P. purum* exposed for 56-day periods in the study area. The values of the indices did not differ between any of the SS, despite these being separated by distances of up to 120 km. The combined variation across these SS indicates the existence of environmental variables that affect the entire study area and generate natural variability in both indices. Finally, from all of the above, we were able to characterize the distributions of the CHL index and PRI and to establish the stress thresholds (1.195 and −0.212, respectively), which correspond to the 5 % percentile of each distribution.

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GENERAL DISCUSSION


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GENERAL DISCUSSION

Bryophytes have been considered good biomonitors of air quality ever since they were first used for this purpose at the end of the 1960s (Rühling & Tyler, 1968). These authors found high concentrations of Pb in moss growing beside roads, and they considered this as evidence of the suitability of moss as a biomonitor. The technique then became known as the “moss biomonitoring technique”. Names always give a certain authority, and without any further testing of the original hypothesis, many researchers during the next 20 years assumed that there was a direct relationship between the elements taken up by moss and the atmospheric deposition of these elements. Although some studies showed that this was only true in certain cases (see *e.g.* Ross, 1990; Berg *et al.*, 1995; Berg and Steinnes, 1997), the assumption was upheld in many other studies. Some factors were even proposed to explain the suitability of moss as biomonitors, *e.g.* the absence of a protective cuticle and of epidermal cells with thick cell walls, and the lack of roots. These characteristics were suggested to confer the moss with a high cation exchange capacity and the ability to capture and accumulate contaminants derived from atmospheric deposition. The suitability of moss as biomonitors was further validated by considering moss as an integrator, an idea maintained in recent studies (see *e.g.* Harmens et al., 2008). These assumptions led numerous authors to try to estimate the absolute rates of deposition of contaminants (*i.e.* metals) from the concentrations in moss tissues (see *e.g.* Rühling *et al.*, 1987; Berg and Steinnes, 1997).

However, up until this point, it had not been considered whether mosses were capable of integrating the contaminants that they received and, therefore, whether they were good biomonitors, as defined by Markert (1999). The conclusions extracted from some studies, in regard to the correlations between the concentration of the elements to which the mosses are exposed and the concentrations in their tissues, were not consistent with the results obtained. Furthermore, little attention was given to the importance of the previously mentioned biological characteristics (*e.g.* absence of cuticle, roots, etc.) in terms of the capacity of mosses to act as biomonitors.

Scarce attention has been given to research on the theoretical bases of the “moss biomonitoring technique”, even in the complex pseudo-technological approach that has culminated in the ICP Vegetation (http://icpvegetation.ceh.ac.uk/), which unites several national moss monitoring networks. Many individuals and centres receive funding to apply this technique, which is fundamentally based on commonly repeated phrases whereby moss is postulated to “act as an integrator”, “reflect levels of atmospheric deposition”, “provide quantitative values”, “take up elements due to its biological characteristics”, etc. Although not based on scientific evidence, these statements are repeated in scientific articles and reports and have become widely accepted. However, many of these ideas have been refuted in recent years. For example, some authors have shown that mosses are not generally capable of integrating the contaminant load that they receive and others have found a direct relationship between the concentration of elements in moss and the total deposition of only some elements (*e.g.* Cd and Pb) (Aboal *et al.*, 2010; Fernández *et al.*, 2013). The research reported in this thesis continues along these lines, with the specific aim of determining whether the concentrations of contaminants in moss should be used to yield quantitative results and whether the uptake of elements depends on the biological characteristics of the moss.

Although some atmospheric contaminants may occur in gaseous form, most are found associated with particles in suspension (Bargagli, 1998). As particles are less disperse than gases, it appears logical to assume that when the “moss biomonitoring technique” is used in the surroundings of focal points of contamination, the highest concentrations of contaminants will be found in the tissues of moss growing closest to these points. Moreover, if the contaminants occur in the gaseous phase, the highest concentrations will be found at distances further from the source of contamination. However, only the first of these assumptions is true. The results obtained with P. purum and reported in Chapter III show that in industrial sites, the concentrations of heavy metals and metalloids decrease directly in relation to increasing distance from the source of contamination, as previously reported (Türkan *et al.*, 1995; Brūmelis *et al.*, 1999; Fernández *et al.*, 2000, 2004; Pöykiö *et al.,* 2001; Čeburnis et al., 2002; Real *et al.*, 2003). However, when the object of study is a gaseous contaminant (*e.g.* N, see Chapter II), the second assumption does not hold true as the concentrations of such contaminants are distributed at random throughout the area affected and do not follow any spatial patterns.

However, the fact that the moss biomonitoring technique appears to be suitable for heavy metals in industrial sites does not imply that moss is capable of integrating all of the contaminant load to which it is exposed or that there is a consistent relationship between the total deposition and the concentrations of elements in the moss. If a large portion of contaminants occur in particulate form in the atmosphere, their uptake in mosses will be affected by the particle dynamics. Particulate matter is bound so strongly to the outer surface of mosses that it is impossible to differentiate this fraction from the fraction that accumulates inside the cells (Spagnuolo *et al.*, 2013). Moreover, far from being inert, the particles interact with the moss and compete with other elements for cation exchange sites (Nieboer and Richardson; 1980 Couto *et al.*, 2004). The research reported in Chapter IV shows that each contaminant displays different binding properties for the exchange sites and that these properties are determined by the ionic and covalent bindings indices. Thus, many of the assumptions that have been made about moss are erroneous. Moss samples cannot yield reliable quantitative data as they do not integrate all of the contaminant load to which they are exposed. Moreover, uptake of contaminants will depend on the physicochemical characteristics of elements and not only on the biological characteristics of the moss, as previously assumed.

For all of the above reasons, researchers must refrain from overrating the potential of moss as a biomonitor and indiscriminately applying the “moss biomonitoring technique” in various situations. Other alternative methods to the chemical quantification of contaminants must be developed. One possible alternative method is physiological biomonitoring, in which the effects of contaminants would be evaluated directly, at physiological or morphological level. As the tissue concentrations of contaminants would no longer be measured, the limitations of the technique (*e.g.* those associated with bioconcentration of the contaminant load, affinity, competition for cation exchange sites, metabolic limitation, etc.) would disappear and the effects caused by the contaminants would become more important, as observed for the moss *P. purum* (Chapter V). Nonetheless, this alternative method would not enable identification of the causal agents of the effects, as it would be impossible to determine all of the chemical compounds present in the moss samples. Use of this type of approach also requires prior measurement of the reference values of the physiological parameters in mosses growing in unpolluted environments to enable evaluation of whether the target moss samples have been affected by contaminants (Chapter VI).

Finally, application of the seriously flawed “moss biomonitoring technique” has led to the development of different methodological approaches in the form of protocols that are not based on scientific criteria. During the past 20 years, the Ecotoxicology research group at the USC has been working towards optimizing these protocols. In this thesis, I report some the results of some of the remaining lines of research. Although delimitation of the size of sampling site may intuitively appear to be key factor, no consensus has yet been reached as regards this point. In most studies, sampling sites of dimensions 50 x 50 m are used, although the choice is not based on scientific findings. The results of this research show that sampling sites of diameter greater than 16 m should be sufficient to detect variations in the concentrations of contaminants. The choice of SS of dimensions 50 x 50 m is therefore valid, although SS of dimensions 20 x 20 would be sufficient.

GENERAL CONCLUSIONS

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1. The size of the sampling site (SS) did not affect the results obtained when using the moss *Pseudoscleropodium purum* to monitor air pollutants; the dimensions of the SS were irrelevant for those elements that displayed spatial independence or linear spatial structure. However, other factors such as the number of subsamples collected and the weight of each can affect the results. For elements that display spatial structure that fits a spherical model, the optimal size of the SS was a circular area of diameter greater than 16 m. However, these conclusions must be verified using the same method with other species and in other regions.

2. The bryophyte *P. purum* cannot be used to biomonitor atmospheric contaminants such as N and some heavy metals and metalloids, for the following reasons:

2.1. Nitrogen is an essential element that is metabolically regulated in moss. The levels reach saturation point and cannot be accumulated further in moss, which cannot therefore comply with the criteria used to define good biomonitors. Futhermore, the concentrations of N were distributed at random throughout the study area and did not display any spatial patterns. Moreover, the concentrations of N in moss were not related to the distance from the sources of emission of this element.

2.2. Mosses can only yield qualitative estimates of the concentrations of heavy metals and metalloids emitted by different industries. They do not integrate all of the contaminant load as most contaminants are retained on the moss surface as particulate material and interact with the moss and compete with other elements for cation exchange sites. The existence of these processes demonstrates that the uptake of contaminants by moss mainly depends on the physicochemical characteristics of the elements and not on the biological characteristics of the moss. This further verifies the inability of moss to yield reliable quantitative data, as it is not possible to determine the bioconcentrated fraction. As the present studies were only carried out with the moss *P. purum*, these conclusions must be verified using other species.

3. Different physiological parameters of the moss *P. purum* (*i.e.* moss growth, chlorophyll fluorescence and spectral reflectance) cannot be used to evaluate air quality, for the following reasons:

3.1. The different processes related to atmospheric contaminants, such as atmospheric deposition, accumulation in moss, physiological parameters, and moss growth are not significantly related. However, the values of the chlorophyll content index (CHL) and moss growth rate both decreased after exposure of moss to high levels of heavy metals and metalloids, suggesting the existence of a threshold-type response for each.

3.2. In the specific case of the CHL index and photochemical reflectance index (PRI), no temporal patterns or differences between study sites were found. The combined variation reveals the influence of the environmental conditions of the study area on these indices. Nonetheless, the distributions of both indices were computed and the corresponding stress thresholds were calculated (1.195 and -0.212 for CHL and PRI, respectively).

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SUMMARY

1. INTRODUCIÓN XERAL

O emprego de briófitas terrestres é unha técnica amplamente utilizada para caracterizar os niveis de contaminantes na atmosfera. A técnica foi desenvolta nos países nórdicos -a finais dos anos 60 do século XX (Rühling e Tyler, 1968; Tyler e Rühling 1970)- como un medio de estudo da deposición atmosférica de metais pesados. As briófitas perfílanse como organismos axeitados para ser empregados como biomonitores por posuír unhas características especiais que lles confire unha a alta capacidade de intercambio catiónico para fixar e acumular contaminantes procedentes da deposición atmosférica. Ademais, posúen a capacidade de reter partículas que se ligan fortemente aos seus tecidos, presentan unha ampla distribución con representación en diferentes hábitats e tamén unha gran facilidade de manipulación no laboratorio cun baixo custo económico. Así, dende que se empregaron por primeira vez, o seu uso estendeuse ao resto de países europeos ampliándose á determinación de diferentes contaminantes tales como: compostos orgánicos (*e.g.* Knulst et al., 1995; Lead *et al.*, 1996; Orlinski, 2002; Gerdol *et al.*, 2006; Carballeira *et al.*, 2006), elementos radiactivos (*e.g.* Sawidis e Heinrich, 1992; Triulzi *et al.*, 1996), non metais como nitróxeno (*e.g*. Harmens et al., 2011) ou halóxenos como fluor (*e.g.* Real et al., 2003).

O éxito da técnica levou a crear un programa europeo de monitorización (The International Cooperative Programme on Effectsof Air Pollution on Natural Vegetation and Crops ICP-Vegetation) da deposición atmosférica de metais pesados, nitróxeno e contaminantes orgánicos persistentes (POPs) mediante o uso de briófitas pertencente ao UNECE (United Nation Economic Commission for Europe). Os obxectivos desta rede de biomonitorización son a obtención dunha caracterización cualitativa e cuantitativa da deposición atmosférica de metais pesados en Europa, a localización das fontes de emisión destes contaminantes, a realización de mapas de patróns rexionais e comparacións retrospectivas entre diferentes campañas.

O grupo de Ecotoxicoloxía da Universidade de Santiago de Compostela leva dende 1995 traballando na biomonitorización da calidade do aire con brións terrestres, fundamentalmente coa especie *Pseudoscleropodium purum* (Hedw.) M. Fleisch. Esta briófita está amplamente distribuído nas rexións mornas do Hemisferio Norte, cunha grande abundancia en Galicia, e é un dos brións máis fáciles para recoñecer no campo e traballar no laboratorio.

Non obstante, e a pesar do eficiente esforzo investigador do grupo (máis de 50 publicacións internacionais nos últimos 15 anos), aínda quedan por estudar aspectos (metodolóxicos e ecofisiolóxicos) para seguir avanzando no emprego de P. purum como biomonitor. O primeiro problema co que nos encontramos é que a día de hoxe, aínda non existe un protocolo harmonizado da técnica do musgo baseado na literatura científica. Séguese empregando o protocolo proposto por Rühling (1989) con mínimas modificacións ata a súa versión actual (Harmens *et al.*, 2005). Un dos factores claves na primeira fase do devandito protocolo é a delimitación da estación de mostraxe para a recolección das mostras de musgo, xa que esta podería determinar os resultados obtidos. Non obstante, ningún protocolo xustifica cientificamente as dimensións da área de mostraxe. Por iso o Capítulo I da presente Tese aborda a necesidade de estandarizar o método comezando por establecer cal debe ser a área de mostraxe ao empregar como biomonitor o musgo *P. purum.* Unha vez que se estudaron as carencias metodolóxicas, o seguinte paso é comprobar a idoneidade de *P. purum* como biomonitor doutro tipo de contaminantes escasamente estudados nos brións, pero con relevancia atmosférica, como é o nitróxeno (Capítulo II). Nas últimas décadas produciuse un aumento das emisións de N á atmosfera en resposta á crecente demanda de alimentos e enerxía do planeta. Isto converteu o N procedente das emisións á atmosfera de distintas actividades humanas na fonte máis importante e dominante nalgúns ecosistemas. Pero ata o momento, os traballos realizados puxeron en evidencia unha débil relación entre a deposición de N e a súa acumulación en tecidos de brións. Unha posible explicación desta débil relación é a regulación do nivel corporal nos musgos; de feito xa se describiu con anterioridade a importancia que ten o N no metabolismo dos briófitos (*e.g.* Koranda *et al.*, 2007; Arróniz-Crespo *et al.*, 2008). Recentemente, como método de biomonitorización alternativo ao baseado na concentración total de N no musgo, formulouse a posibilidade de determinar o contido relativo dos seus isótopos estables (^{14}N e ^{15}N) mediante a relación δ^{15}N (Zechmeister *et al.*, 2008).

Así mesmo, o alto grao de industrialización das últimas décadas, fai necesario establecer un instrumento que permita caracterizar as emisións atmosféricas, que facilite seguir a súa evolución temporal e que sirva de axuda á mellora do comportamento ambiental por parte da industria. O sector industrial é o causante principal das emisións de metais pesados e metaloides á atmosfera. Existen inventarios oficiais de emisións como o European Pollutant Releaseand Transfer Register (L-PRTR; http://prtr.ec.europa.eu/) que subministran información sobre a contaminación emitida polas principais plantas industriais da Unión Europea, Islandia, Noruega, Liechtenstein, Serbia e Suíza. Non obstante, na maioría dos casos os datos dispoñibles proceden en maior medida de estimas ou cálculos que de medicións directas das emisións. Isto pode dar lugar a que determinadas industrias emitan máis contaminantes dos que aparecen rexistrados no inventario, o que podemos considerar como: falsos negativos. Fronte a esta situación, é necesario saber se a biomonitorización con *P. purum* evitaría estes casos de falsos negativos para metais pesados e metaloides e se permitiría a elaboración dun inventario de emisións complementario aos rexistros oficiais (Capítulo III).

Aínda que tradicionalmente o musgo sempre se considerou un biomonitor ideal para estimar a deposición atmosférica de metais pesados e metaloides, autores como Aboal *et al.* (2010) apuntan que, en ocasións, o musgo non reflicte axeitadamente os niveis atmosféricos de certos elementos probablemente debido as características fisicoquímicas dos contaminantes e os procesos fisicoquímicos nos musgos. Por iso é inevitable investigar en qué medida a carga de elementos no brión *P. purum* é un fenómeno dependente das características fisicoquímicas destes (Capítulo IV). Porque a pesar da gran cantidade de estudos de biomonitorización que emprega o musgo, os mecanismos fisicoquímicos concretos dos procesos de bioabsorción son aínda bastante descoñecidos a diferenza do que acontece para outros organismos (González e Pokrovsky, 2014). De feito, en ningún estudo da bibliografía dispoñible se investigou a posible influencia das características fisicoquímicas dos contaminantes (*e.g.* cantidade de contaminante e forma de emisión)e dos procesos fisicoquímicos nos musgos (*e.g.* afinidade dos contaminantes polos lugares de intercambio catiónico, competencia dos metais ou metaloides polos lugares de intercambio catiónico, sinerxismo na captación de contaminantes) na carga dos diferentes contaminantes atmosféricos.

Á parte da biomonitorización química, que simplemente nos informa da concentración do contaminante no musgo, é conveniente estudar vías de biomonitorización alternativas como por exemplo a biomonitorización con parámetros fisiolóxicos. É dicir, en vez de determinar contaminantes atmosféricos nos biomonitores, avaliar directamente os efectos que estes producen nos devanditos organismos tanto a nivel fisiolóxico coma sobre o seu crecemento. Estes posibles efectos estudáronse en poucas ocasións (Tuba *et al.*, 1997; Tremper *et al.*, 2004; Aboal *et al.*, 2008) polo que o Capítulo IV pretende abrir unha liña novidosa de investigación e observar as relacións entre os sucesivos procesos relacionados con estes contaminantes atmosféricos como son a súa deposición atmosférica, os seus niveis en *P.purum*, a resposta de parámetros fisiolóxicos -como o Índice de Contido en Clorofilas (CHL; Lichtenthaler *et al.*, 1996) ou a eficiencia fotosintética Fv/Fm (Bolhàr-Nordenkampf *et al.*, 1989)-, e por último o seu efecto sobre o crecemento do musgo.

Non obstante, non existen estudos que determinasen os valores de referencia (albores) destes parámetros fisiolóxicos en musgo, é dicir, en situación de non estres fisiolóxico producido por contaminación, para poder interpretar axeitadamente os resultados obtidos. Por esta razón, no último capítulo da Tese (Capítulo V), mediante a utilización de transplantes do brión *P. purum* preténdese, en primeiro lugar, establecer os valores albores para os índices CHL e de Reflectancia Fotoquímica (PRI) (Fillela *et al.*, 1996) e, en segundo lugar, estudar os seus patróns temporais e a súa variabilidade rexional. As futuras investigacións centraranse en caracterizar e establecer os valores de referencia da ratio Fv/Fm debido a que neste último estudo non se dispoñían de datos para o devandito parámetro.

2. OBXECTIVOS XERAIS.

1. Propoñer un método para determinar o tamaño óptimo da área de mostraxe na técnica do musgo coa especie *Pseudoscleropodium purum* baseado na estrutura espacial dos contaminantes das submostras recollidas en diferentes estacións de mostraxe en Galicia e aplicalo ás concentracións de As, Cd, Cu, K, Hg, Ni, Pb, Se e Zn.

2. Verificar a idoneidade de *P.purum* como biomonitor da contaminación atmosférica comprobando (Capítulo II, III e IV):

2.1. Se é un bo biomonitor do N atmosférico a través da concentración corporal de N total ou da relación isotópica $\delta^{15}N$ mediante a confirmación das seguintes hipóteses: i) non hai diferenzas interespecíficas nas concentracións de N total nin de $\delta^{15}N$ entre as dúas especies de musgo terrestre máis utilizadas, e ii) non hai estrutura espacial nas concentracións totais de N ou nas concentracións de $\delta^{15}N$ nos arredores de fontes de emisión de N a grande ou pequena escala.

2.2. Se permite caracterizar as emisións de metais pesados e metaloides en ámbitos industriais e facilitar a elaboración dun inventario de emisións mediante: i) determinación de calquera modelo de emisións de contaminante para sectores industriais individuais, ii) comparación de calquera de tales modelos con modelos previamente descritos, e iii) establecemento dun inventario dos metais pesados e metaloides emitidos polas industrias consideradas.

2.3. En qué medida a carga dos elementos nos musgos é dependente das súas características fisicoquímicas.

3. Estudar en qué medida as respostas fisiolóxicas observadas en trasplantes de *P. purum* permiten utilizar a técnica de biomonitorización activa para a caracterización da calidade do aire (Capítulo V e VI):

3.1. Determinación de calquera relación entre as concentracións de elementos na deposición total e no musgo, en parámetros fisiolóxicos e no crecemento do musgo, e determinar se algunha distas relacións están afectadas pola duración da exposición ou o nivel de contaminación.

3.2. Investigación da existencia de patróns temporais, variabilidade rexional e valores de referencia do Índice de Reflectancia Fotosintetica (PRI) e do Índice de Contido en Clorofilas (CHL) no brión terrestre *P. purum*.

3. OBXECTIVOS, RESULTADOS PRINCIPAIS E CONCLUSIÓNS DE CADA CAPÍTULO.

CAPÍTULO I :

Determinación do tamaño óptimo de área da mostraxe na técnica de biomonitorización con musgo.

O obxectivo do presente estudo foi determinar o axeitado tamaño da área de mostraxe na técnica do musgo, en función da variación espacial das concentracións dos contaminantes bioconcentrados a pequenas distancias (<30 m). Para iso comprobouse a existencia de estrutura espacial nas concentracións de varios metais e metaloides mediante a realización de semiovariogramas calculados mediante un estimador robusto da varianza. Así, en cada unha das 3 estacións de mostraxe (EM) obxecto de estudio (nunha delas se tomaron mostras en dúas ocasións), recolectáronse 50 submostras do brión *Pseudoscleropodium purum* das que se determinaron as concentracións de As, Cd, Cu, Hg, K, Ni, Pb, Se e Zn. Os resultados obtidos mostran que na maior parte dos casos estudados, 25 de 32, os elementos non presentan estrutura espacial, e queda sometido todo o semivariograma o "nugget effect". Para os elementos que presentan independencia espacial, ou unha estrutura espacial lineal, o tamaño da área de mostraxe non condiciona os resultados obtidos sendo as dimensións de ditas áreas irrelevantes dentro do rango estudado. A existencia dunha estrutura espacial con axuste esférico permitiu definir un rango de autocorrelación que permite determinar o tamaño de área da área de mostraxe, que correspondeuse cun área superior a 16 m de diámetro. O método proposto debería ser aplicado a outras especies e en outras rexións para harmonizar o tamaño da estación de mostraxe a empregar na técnica do musgo.

CAPÍTULO II :

Uso dos musgos epixeos como biomonitores da deposición atmosférica de Nitróxeno.

No presente traballo comprobouse se o brión terrestre *Pseudoscleropodium purum* pode usarse como biomonitor da deposición de N atmosférico. Primeiramente, estudouse se existen diferenzas interespecíficas para as concentracións de N total e $\delta^{15}N$, entre dous das especies de bFións terrestres máis utilizadas en biomonitorización, *Pseudoscleropodium purum* e Hypnum cupressiforme. En segundo lugar, determináronse os patróns espaciais de N e 15 N a pequena e grande escala: i) en 165 mostras ao redor dunha fábrica de alumina e ii) en 149 estacións de mostraxe pertencentes a unha rede de mostraxe regular de 15 x 15 km situada en Galicia (NO España). Non se detectaron diferenzas interespecíficas entre as especies estudadas nin para N total nin para $\delta^{15}N$. Non obstante, si se detectou que o $\delta^{15}N$ permite identificar os patróns espaciais a grande escala xerados polas fontes de emisión de N a diferenza do que acontece empregando o N total, e que en determinadas circunstancias reflicten os patróns por focos de contaminación a pequena escala. Con estes resultados pódese afirmar que o $\delta^{15}N$ presenta unha vantaxe respecto ao N total que é informar sobre a procedencia do N e non sobre a cantidade de N recibido. Esta vantaxe é debida a que o N é un macronutriente que afecta á fisioloxía dos briófitos a diferenza do $\delta^{15}N$, que non se atopa afectado nin pola regulación metabólica nin pola existencia de saturación na súa acumulación.

CAPÍTULO III :

Uso dun método de biomonitorización con musgos para elaborar inventarios de emisións de industrias contaminantes a pequena escala.

O método para detección de fontes de polución a pequena escala (DSSP) proposto por Fernández *et al.*. (2007) foi aplicado para: i) analizar se existen patróns de emisión de contaminantes propios de cada sector industrial, ii) comparar eses patróns cos descritos na bibliografía e iii) obter un inventario de metais pesados e metaloides emitidos polas industrias. O "cluster" análise indica claramente que non existe un patrón común a todas industrias do mesmo sector probablemente debido a diferenzas no tipo de fuel empregado, as materias primas ou no proceso produtivo. Non obstante, cando diversas fábricas comparten estas variables, a determinación das concentracións elementais no musgo permitiu a súa agrupación. Elaborouse un inventario de metais pesados e metaloides para as industrias estudadas e só en 3 dos 56 casos estudados as limitacións do método DSSP impediron a súa correcta aplicación. Por iso, o método convértese nunha ferramenta para a elaboración de inventarios oficiais que tamén pode ser empregado para determinar a efectividade de melloras técnicas aplicadas a unha industria respecto á redución da contaminación ao longo dos anos como para deseñar plans de seguimento e vixilancia ambiental.

CAPÍTULO IV :

Influencia das características fisicoquímicas dos contaminantes no proceso de captación por parte do musgo.

As briófitas son comunmente empregados como biomonitores para estimala deposición atmosférica de metais pesados e metaloides, non obstante hai ocasións nas que o brión non reflicte axeitadamente os niveis atmosféricos de certos destes elementos. Este estudo pretende investigar se a carga de elementos no musgo relacionase coas súas características fisicoquímicas. Por iso mediante análises factoriais preténdese buscar patróns de covariación na acumulación de elementos no brión *P. purum* recollido no ámbito de diferentes industrias e en lugares con niveis de fondo. Os resultados mostran a existencia dun patrón común de variación das concentracións de elementos no musgo, presente tanto en mostras de zonas industriais como de niveis de fondo e unha relación coas propiedades de enlace deses elementos, o que suxire que as características fisicoquímicas dos elementos teñen unha influencia determinante no proceso de captación de metais e metaloides provenientes da atmosfera. A consecuencia principal deste fenómeno é que os estudos que empregan correlacións múltiples entre elementos como unha forma de determinar a orixe común dos contaminantes poderían obter conclusións erróneas ó pasar por alto as propiedades de absorción do musgo.

CAPÍTULO V :

Respostas fisiolóxicas e de crecemento aos contaminantes atmosféricos en transplantes Pseudoscleropodium purum.

A biomonitorización con brións terrestres é unha ferramenta de vixilancia ambiental das actividades antropoxénicas que liberan ao medio un gran número de compostos químicos contaminantes. Este estudo pretende avaliar os efectos que estes compostos producen no brión a nivel fisiolóxico, e que van ser o resultado dunha serie de procesos sucesivos relacionados cos contaminantes presentes na atmosfera: emisión- inmisión- captación do musgo (carga total)- bioconcentración no musgo- alteracións fisiolóxicas – respostas o dano. Realizáronse transplantes de musgo ao redor dunha aceiría en 5 estacións de mostraxe a distancia crecentes dende a industria e para 8 períodos de tempo distintos: 4 períodos mensuais, 2 bimestrais e 1 de 4 meses. Posteriormente, determináronse as concentracións de Al, As, Cd, Cr, Cu, Fe, Hg, Ni, Pb, V e Zn, Índice de Contido de Clorofila(CHL), a eficiencia fotosintética (Fv/Fm) e o crecemento do musgo, cos obxectivos de: (i) Determinar se existe relación entre os sucesivos procesos da serie: deposición total, concentracións no musgo, parámetros fisiolóxicos e crecemento do musgo; (ii) Determinar se o tempo de exposición e o nivel de contaminación altera as relacións anteriores en caso de que existan. Os resultados mostraron que non existe relación entre os sucesivos procesos da serie aínda que se se observa un descenso de "CHL" e crecemento con altos niveis de metais e metaloides determinados no musgo, o que permite hipotetizar a existencia dunha resposta de tipo albor para ámbalas dúas variables.

CAPÍTULO VI :
Establecemento dos valores de referencia dos Índices de reflectancia espectral en transplantes de Pseudoscleropodium purum para o seu uso biomonitorización atmosférica.

Establecéronse os valores de referencia para o índice de reflectancia fotoquímica (PRI) e o Índice de contido de clorofila (índice CHL) para uso en biomonitorización contaminación atmosférica na base de estres fisiolóxico en musgos terrestres. En ámbolos dous índices, determinouse a: i) a existencia de patróns estacionais, ii) variabilidade rexional e iii) límites de referencia. Non se detectaron patróns ou diferenzas estacionais entre os diferentes sitios na zona de estudo, e a variación entre as estacións reflectiu a influencia de variables ambientais na zona. Establecemos os limiares de estres como -0.212 para “PRI” e 1.195 para “CHL”.

4. CONCLUSIÓNS XERAIS.

1. O tamaño da area de mostraxe non afecta os resultados obtidos cando se emprega o brión *Pseudoscleropodium purum* na monitorización dos contaminantes do aire.; as dimensións desta area foron irrelevantes para os elementos que mostraron independencia espacial ou estrutura espacial lineal. Non obstante, outros factores como o número de submostras recollidas e o peso equilibrado entre as mesmas si que poderían influír nos resultados. Para elementos que presentan estrutura espacial con axuste esférico comprobouse que o tamaño da estación corresponde a un área cun diámetro maior a 16 m. Aínda así estas conclusións deberían ser verificadas noutras especies e rexións empregando a mesma metodoloxía.

2. A briófita *P. purum* non pode ser considerado como biomonitor axeitado de contaminantes atmosféricos como Nitróxeno e algúns metais pesados e metaloides xa que:

2.1. O N é un macronutriente regulado metabólicamente polo musgo que presenta saturación na súa acumulación non cumprindo así a definición dun bo biomonitor. Por unha parte, as súas concentracións teñen unha distribución aleatoria ao largo do territorio estudado e non mostran ningún patrón espacial. Ademais, no entorno de focos de emisión de este elemento non hai ningunha relación entre as concentracións no musgo e a distancia aos mesmos.

2.2. Os musgos so permiten caracterizar as concentracións de metais pesados e metaloides emitidos á atmosfera por diferentes industrias de forma cualitativa. Non integran toda a carga contaminante á que están expostos xa que a maioría dos contaminantes quedan retidos na superficie do musgo como material particulado sufrindo procesos de interacción co musgo e competencia por lugares de intercambio catiónico. A existencia destes procesos demostra que a carga de contaminantes por parte do musgo depende fundamentalmente das características fisicoquímicas dos elementos e non das súas características biolóxicas, verificando que o musgo non pode aportar valores cuantitativos fiables porque descoñecese a fracción que pode ser bioconcentrada. Dado que estes estudos realizáronse co musgo *P. purum* sería necesario verificar estas conclusións empregando outras especies.

3. Diferentes parámetros fisiolóxicos do musgo *P. purum* (*i.e.* crecemento do musgo, fluorescencia clorofílica e reflectancia espectral) non poden ser empregados para a caracterización da calidade do aire xa que:

3.1. Non hai relacións significativas entre os sucesivos procesos relacionados cos contaminantes atmosféricos como son deposición atmosférica – concentración no musgo – parámetros fisiolóxicos – crecemento do musgo. Aínda que para o “chlorophyll content index (CHL)” e crecemento do musgo si que se observou un descenso nas mostras con niveis elevados de metais e metaloides, o que permite hipotetizar a existencia dunha resposta de tipo albor para ambos.

3.2. No caso concreto dos parámetros “CHL” e “índice de reflectancia fotoquímica (PRI)” non existen patróns temporais nin diferenzas entre as estaciones estudadas. A variación conxunta de ambas revela a influencia que teñen sobre elas as condicións ambientais da zona de estudio. Aínda así caracterizáronse as distribucións de ámbolos dous índices e calculáronse os valores de referencia de estres correspondentes (1.195 e -0.212 para CHL e PRI, respectivamente).

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A Rubén Retuerto

Ya sé que vas a decir que tú no hiciste nada pero es mentira! Siempre has estado ahí! Cualquier duda, consejo o cosa que necesitara…solo tuve que llamar a tu puerta! Gracias!

ECO

Tere

I've got another confession to make, I'm your fool…everyone's got their chains to break holdin' you... Were you born to resist or be abused? Is someone getting the best, the best, YOU'RE THE BEST, the best of you???

Angy

I, I will be King and you, you will be Queen…Though nothing, will keep us together we could steal time, just for one day *we can be heroes*, for ever and ever…

Marta

Lunes, martes, miércoles *cotilleando* sin parar, el lab en un buen lugar para irse a *rajar*… …Jueves, viernes, sábado *sentadas* en el sofá, es un buen lugar para irse a *olvidar*, en Santiago aún no se huele el sooooool… No hables de f*uturo* es una ilusión cuando *ecotox* conquistó *nuestro* corazón…..!!!!

Mamá-María

Las peras caen maduras…!

Marga

El que resiste, GANA!

Sofi

Dicen que "Un consejo es algo que pedimos cuando ya conocemos la respuesta, pero quisiéramos no conocerla"….te seguiré pidiendo consejos *siempre* Señorita Fontinallis…

Carlitos

Como bien sabes Brais…"la pregunta es importante solo si la respuesta es correcta"…así que... Tú cómo te llamas??? Conejito, y tú??? Tigrito…. No, no mi grites! :-(jaaaaaaaajajajajajaja

Sergio

And now, the end is near and so I face the final curtain, *my friend* I'll say it clear….*I did it my waaaaaaaaaaaaaaayyy!*

También a los que participaron de una u otra manera en esta tesis, ayudando en el laboratorio o simplemente dándome ánimos: Xabi, Cris Iglp, Rai, Ana Rey, Anita Corredoira, Irene, Rita... Y por supuesto a los que ya no están pero estuvieron: Johanna, Sarita Pocahontas, Borja, Alicia....

OU

Luis

Maggie May told me that WE ARE BORN TO RUN aunque perdínme polo camiño na primeira parte contratante da segunda parte considerada como a parte contratante da primeira parte porque alguén berraba TRAED MADERA! ES LA GUERRA! E so quería que *how I wish, how I wish you were here* entón dinme conta de que *you can't always get what you want* pero ó final o comprendín: HASTA LA VICTORIA SIEMPRE.

Susi

Si volviera *nacer*, si empezara de nuevo, volvería a nacer en la misma casa de *cuento*. Son las *madres* quiénes nos *llevan* y nos *guían*…. a través de la *vida*…

A Re, Fa, Bei y Jaime (+1)

Telleira + Vacaciones + Cumpleaños + Sobaos + Discusiones + Risas + Tronco de Navidad + Prioridades = FAMILIA.

Evouski:

Thank you Mario! But your princess is in another castle!

A los Varela-Parada

¿Qué culpa tengo yo de tener la sangre roja y el corazón a la izquierda??? (Che Guevara)

A LA CUCHIPANDI

Rebe

When you try your *best*, but you don't *succeed*, when you get what you *want*, but not what you *need*… lights will guide your home and ignite your bones and YOU ALWAYS TRY TO FIX ME. I hope you don't mind that I put down in words *how wonderful life is while you're in the world…*

Camilo

G= H-TS, ΔG=0 → El equilibrio termodinámico de un sistema *amistoso* se define como la condición del mismo en el cual las variables empíricas de amistad usadas para definir o dar o conocer el estado del sistema (risas, bici, risas, caminatas, risas, verano, risas, camping, risas, bolos de Caxide, risas, teorías absurdas, risas, festas, risas….) *no son dependientes del tiempo*…

Lore

Me gustan los periodistas/ fotógrafos me gustas tú, me gusta pasear me gustas tú, me gusta ir de pinchos me gustas tú, me gusta Noa me gustas tú…..!!! ♪♪ Minha torcida minha querida minha galera o minha cachoeira minha MENINA…♪♪♪

Jairo

A quién le importa lo que yo haga? A quién le importa lo que yo diga? *yo soy así*, y así seguiré *nunca cambiaré*… por eso… And if I only could make a deal with god and get him to swap our places BE RUNNING UP THAT HILL...

A Pau Nesky

Zumo tells you that "I *never* had much *faith* in love or *miracles*….never wanna put my *heart* on deny… cause you make feel like, I've been locked out of *heaven*…for too long, for too looooooooooong".

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Alba

Un tal Cicerón dixo…¿Qué cousa máis grande que ter a alguén con quen te atrevas a falar como contigo mesmo?

Marta Abajo

ADAPTARSE O MORIR!!!!

Laura Fachal

So close no matter how far, couldn’t be much more from the heart, *forever trust in who we are*…AND NOTHING ELSE MATTERS.

Inés

You soft and only, you lost and lonely you, strange as angels, dancing in the deepest *ocean*, twisting in the water….you…. *just like heaven*!

Merche

-Quixera que sempre fose así- dixo Bastian
-“Sempre” é so un momento- respondeu a Emperatriz Infantil...

Os de “Mellora”: mens sana in corpore sano…

VIGO

Hei, Raqui y Rob

Esperad mi llegada con la primera luz del 5º día, al alba, *mirad* al este….. (a no ser que tenga retención en Puxeiros y tarde un poco más… jaaaaajaja)

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"La paciencia es un árbol de raíces amargas pero de frutos muy dulces."

//Maquetación y Diseño //
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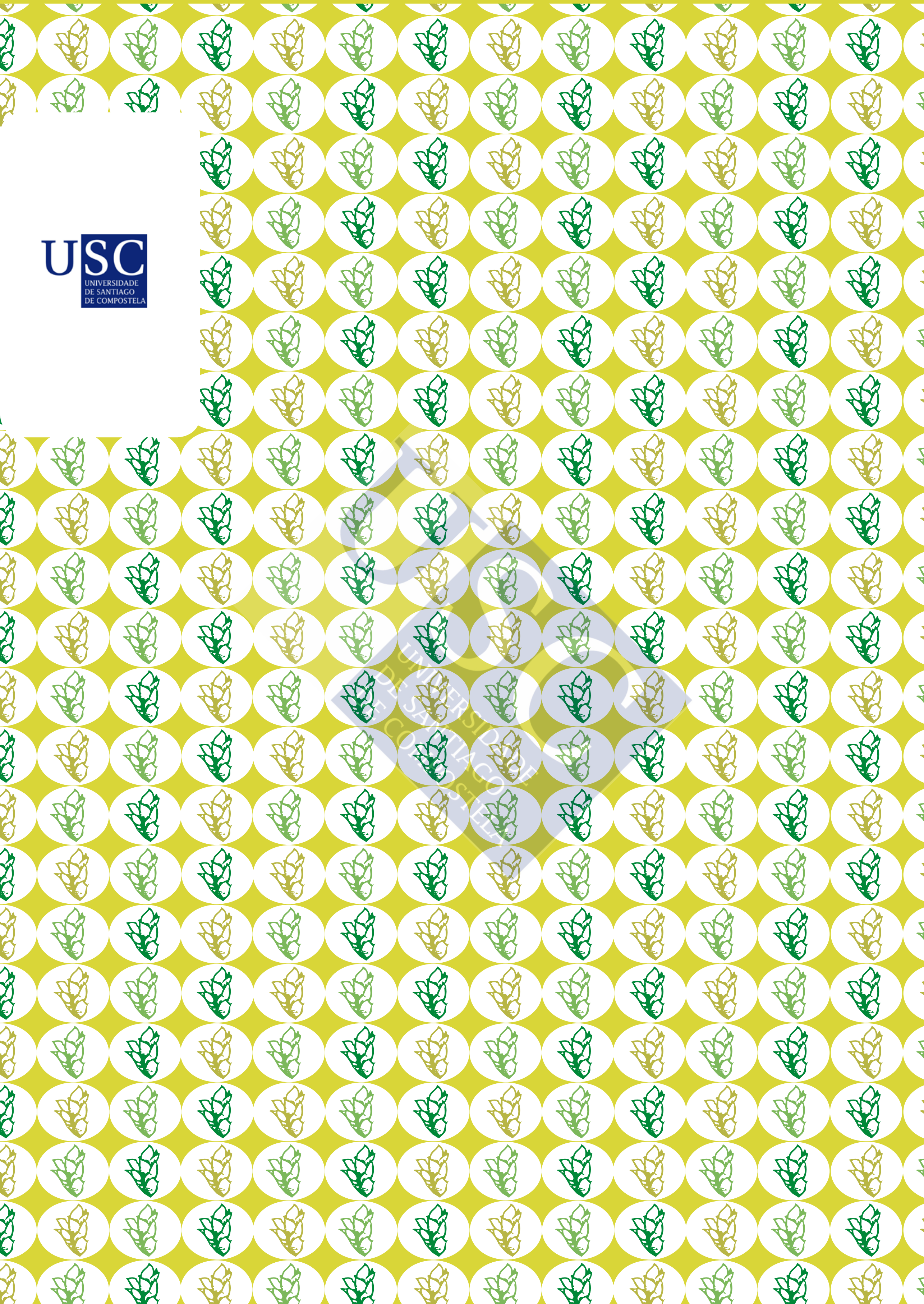

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