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ÁREA DE ECOLOGÍA

A critical evaluation of the use of the moss technique to monitor air pollution

Memoria que presenta
Mª Teresa Boquete Seoane
para optar al Grado de Doctora en Biología

Fdo: **Mª Teresa Boquete Seoane**
Santiago de Compostela, Diciembre 2014

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CERTIFICAN:

Que la presente memoria titulada **"A critical evaluation of the use of the moss technique to monitor air pollution"** presentada por Dña. **Mª Teresa Boquete Seoane** 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 a 16 de Diciembre de 2014.

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A mi madre, a mis herman@s

y a Ibán

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GENERAL INTRODUCTION

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General Introduction GI

1.1. Release of pollutants into the atmosphere

Trace elements (found at low levels in biological systems), and more specifically metals and metalloids (e.g. Cd, Co, Cr, Cu, Hg, Mn, Pb and Se), have always entered the atmosphere naturally. Events and sources such as volcanic eruptions, wild forest fires, wind-borne soil particles and sea spray have episodically or continuously led to the input of trace elements into the air. However, emission of these elements has increased considerably due to human activities, as a result of technological advances and demographic and economic growth over the last centuries (particularly since the Industrial Revolution). Nowadays the most important anthropogenic sources of emission include mining and smelting of metallic ores, industrial production, combustion of fossil fuels, incineration of refuse and cement production (Freedman and Hutchinson, 1981; Bargagli, 1998).

Once pollutants enter the atmosphere, they can be rapidly deposited close to the source of emission or transported over long distances through the atmosphere. Which of these occurs depends on the physical and chemical characteristics of the pollutants (e.g. particle size, chemical form), the characteristics of the source of emission (e.g. height of the stack, composition of the pollutant mixture) and on environmental factors (e.g. winds, precipitation events) (Treshow, 1984). However, trace elements will eventually be removed from the atmosphere and reach the earth's surface. This may occur via dry or wet deposition. The former involves the physical removal of particulate matter by processes such as gravitational settling, turbulent diffusion, Brownian diffusion and impaction. The relative importance of each of these processes will primarily depend on the size of the particles (i.e. coarse particles will mainly be deposited by gravitational settling). Dry deposition is particularly important in arid and semiarid regions where removal by wet deposition is low due to limited precipitation. Wet deposition consists of the removal of elements together with some form of precipitation (i.e. rain,

snow and hail). This also includes the so-called occult deposition (fog and mist) (Treshow, 1984; Injuk and Grieken, 1995). These mechanisms probably constitute the routes whereby the atmosphere tends to be restored to an unpolluted state by natural processes (Purves, 1977). Nevertheless, this implies the transfer of hazardous and persistent elements to the earth's surface where they may interact with living organisms.

Some trace elements are considered essential for living organisms as they play important roles in metabolic processes (i.e. Co, Cr, Cu, Fe, Mn, Mo and Zn). For example, Zn can form stable bonds with proteins, acting as catalyst by enhancing enzyme activities, and Cu is involved in photosynthesis and protein and carbohydrate metabolism. Elements such as Fe and Mo, which have a stable oxidation state that differs only by one unit (Fe^{+2} - Fe^{+3}, Mo^{+5} - Mo^{+6}), are involved in electron transfer processes (Bargagli, 1998). However, some elements can become toxic at high concentrations (i.e. Co, Fe and Mo), or the range between deficiency and toxicity symptoms may be very narrow (i.e. Cu). Other elements have no known function in living organisms and are toxic even at very low concentrations (i.e. Cd, Hg, Pb). The toxicity may involve structural damage, abnormal functioning of cell metabolism or reduced growth at an individual level (Puckett 1976; Richardson et al., 1979), but may also involve changes in structure at population and community levels (Folkeson, 1979). The bioaccumulation index of elements such as Cd, Co, Cr, Cu, Hg, Pb, Ni and Zn is moderate to high (Treshow, 1984), and therefore their transfer through the food chain is likely. The inclusion of these elements in surface ecosystems constitutes a very important environmental and health problem.

1.2 Measuring air pollution: classical vs. new methods

The negative impact of trace elements on living organisms (plants, animals and/or humans) has led to increasing concern in modern society about obtaining information on the quality of the environment. This information can be obtained by monitoring, which in the case of air quality consists of the measurement of the levels of pollutants released into the air. Such mea-

surements, together with the assessment of the effects of pollutants in the biosphere, would help governments and policy makers control air pollution and establish policies and regulations aimed at protecting the environment. Routine measurements (such as those carried out as part of monitoring networks) would also serve as early warning systems to prevent negative effects of pollutants on natural ecosystems and would provide information about the spatial and temporal changes in pollutant concentrations.

Two approaches used to monitor air pollutants can be distinguished. Both show advantages and disadvantages relative to each other, and both are complementary to one another. The first approach involves the use of "classical methods" involving the physicochemical analysis of precipitation, particles and gases. These are commonly described in regulations (i.e. Directive 2004/107/EC relating to arsenic, cadmium, mercury, nickel and polycyclic aromatic hydrocarbons in ambient air; Council Directive 1999/30/EC relating to limit values for sulphur dioxide, nitrogen dioxide and oxides of nitrogen, particulate matter and lead in ambient air), and they are quite standardized. Use of these methods provides absolute heavy metal deposition values; nonetheless, the measurements require the use of very expensive equipment, which must also be maintained, and the number of sampling sites that can be established is therefore very limited (implying monitoring networks of low spatial resolution). Moreover, classical measurement techniques are subject to serious problems of contamination because of the low levels of pollutants in the air, and the data obtained are therefore not as reliable as desired (Bargagli, 1998; Wolterbeek, 2002).

On the other hand, it is also possible to use living organisms that respond in some way to the levels of pollutants that they receive from the atmosphere. Assessment of the response provides valuable information about the characteristics of the air under which the organism completes its life cycle. This method is known as biological monitoring and has proven very useful when physicochemical observations are not sensitive enough (which would imply no sign of alteration when it actually exists) or are too expensive for frequent measurements to be made (which, for instance, would led to the loss

of information about brief or intermittent pollution events). When biological monitoring is based on the assessment of qualitative variables (e.g. presence/absence of an organism or changes in abundance), it is known as optical bioindication, and the organisms used are called bioindicators. When it is based on the measurement of quantitative variables (e.g. levels of pollutants in the organism), it is known as biomonitoring, and the organisms involved are called biomonitors. Figure 1 (adapted from Phillips, 1980) shows the relationship that a potential bioindicator (blue squares) or biomonitor (green squares) should maintain with the pollutants to which it is exposed (in this case for metal inputs). Thus, an organism present only in polluted or in unpolluted areas would be considered a potential bioindicator. An organism capable of accumulating pollutants, the concentrations of which are directly related to the concentrations of the pollutant in the surrounding environment, constitutes a potential biomonitor.

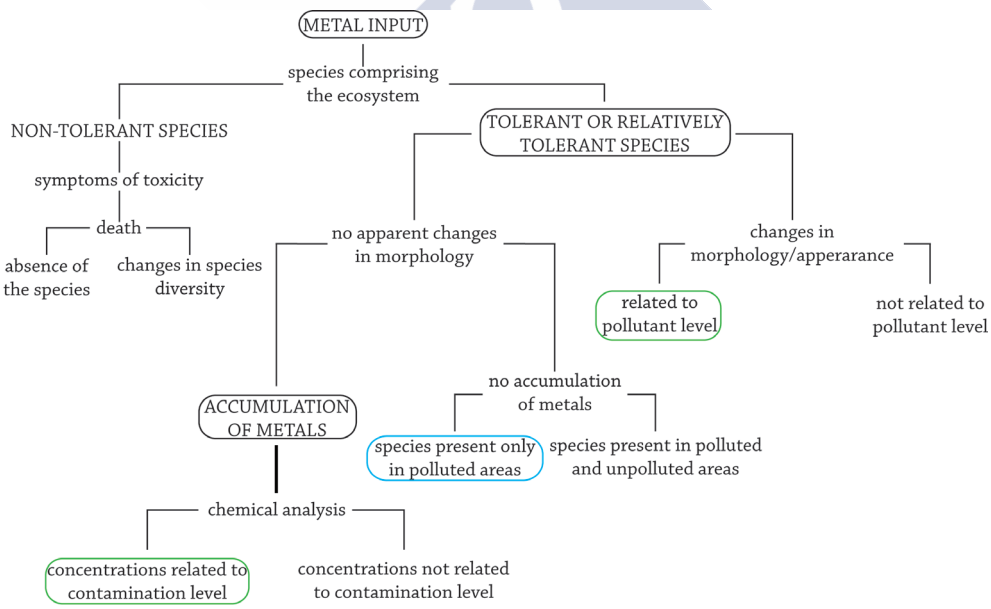


Figure 1: Relationships established between pollutants (in this case metals) and living organisms. Green squares: potential biomonitor organisms; blue squares: potential bioindicator organisms. (Modified from Phillips, 1980).

1.3 New methods: passive and active biomonitoring of air quality

The interest in biomonitoring to assess air quality has increased considerably in modern society because it constitutes a simpler, cost effective and more reliable approach than classical methods (concentrations in the biomonitor organism are higher than in the air leading to lower analytical errors and less contamination problems). Although this approach does not provide absolute deposition values, it does enable identification of polluted areas. Furthermore, it enables characterization of the spatial and temporal patterns of the atmospheric deposition of trace elements, most importantly, when regional, national or transnational patterns must be assessed. This approach has even been considered in the legislation (Directive 2004/107/EC, also called the Fourth Daughter Directive).

Two types of air quality biomonitoring can be distinguished: passive and active (for more details, see e.g. Markert et al., 2003). The first involves collection of the biomonitor directly in the environment where it grows, while the latter is based on the use of transplants of the biomonitor organism (prepared in the laboratory), which are then placed in the area to be studied (under controlled conditions). The use of transplants (active biomonitoring) has some advantages, as it overcomes the following problems related to passive biomonitoring: i) absence of the biomonitor organism in heavily polluted environments (i.e. industrial areas, urban settlements), or in areas where the climatic conditions do not favour its presence (Giordano et al., 2009); ii) development of phenotypic or genotypic adaptations of the organism to the toxicity caused by pollutants (Bargagli, 1998); iii) absence of environmental stressors that affect moss uptake processes (e.g. dehydration of tissues during drought periods); and iv) standardization of the time of exposure. Thus, transplants are exposed during a known and well defined period of time while the period of exposure of native biomonitors will depend on their growth rates, which are not always well known. However, passive biomonitoring still constitutes a very valuable tool as it enables extensive monitoring networks (including national and even transnational networks)

to be established at a very low cost (which would not be viable in the case of active biomonitoring).

1.4 Passive biomonitoring: selection of the biomonitor organism

One of the key steps in a biomonitoring study is selection of the biomonitor species, as not all organisms can be used to monitor air pollution. Some of the requirements of candidate biomonitors include the following (Chakrabortty and Paratkar, 2006): i) a wide geographical distribution (the organism should be common in the area of interest, whether a small area, a region or the entire country); ii) accessibility, low mobility and good availability in all seasons; iii) easy identification and handling; iv) sufficient biomass for the chemical analysis; v) limited biological variability (to reduce intraspecific differences in element accumulation capacity); vi) tolerant to measurable levels of pollutants (if not, organisms will die in heavily polluted environments); vii) uptake of elements independent of specific local conditions, so that the concentrations of the pollutant in the organisms' tissues are directly correlated with the concentrations in the environment; viii) the pollutants accumulated in the tissues should not come from sources other than atmospheric deposition.

Among living organisms, plants (both higher and lower plants – see Fig. 2) are probably the most useful biomonitors for atmospheric deposition of trace elements as they are directly exposed to airborne pollutants (they obtain some of their nutrients from the atmosphere), they are not mobile, and they usually have longer life cycles than animals (Bargagli, 1998). In the case of higher plants, leaves or needles (Alfani et al., 1995; Bussotti et al., 1997; Ukonmaanaho et al., 1998) and tree bark (Panichev and McCrindle, 2004) have often been used to monitor airborne pollutants in industrial, urban and rural environments because these parts are directly exposed to atmospheric deposition but are less exposed than other plant parts to contamination from soil and splashing. In the case of lower plants, bryophytes (particularly mosses) and lichens are the most common organisms used as biomonitors. Both of these types of plants are able to survive in a wide range

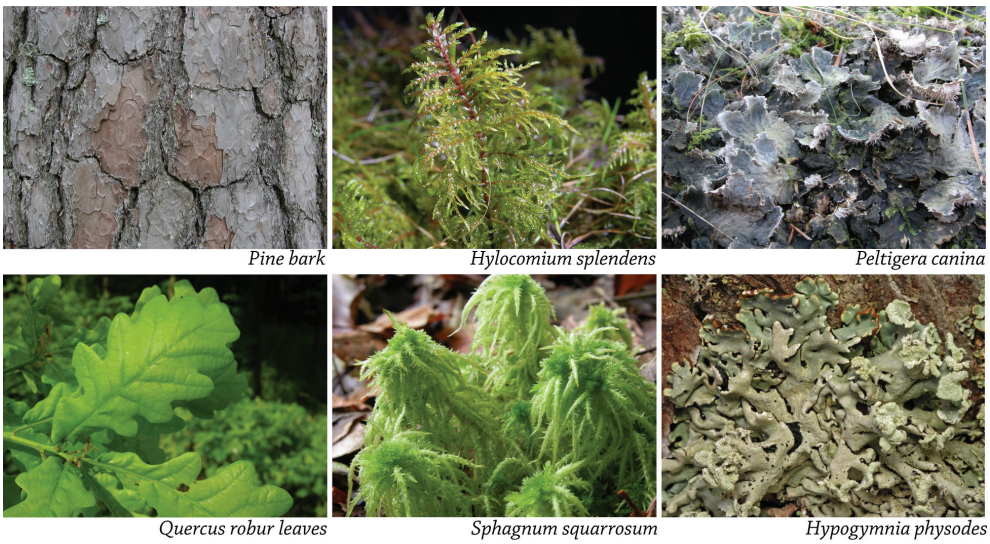


Figure 2: Examples of organisms/materials frequently used in biomonitoring studies. A) Higher plants (leaves and bark); B) terrestrial mosses; and C) lichens (all photos were taken from www.biopix.es, except that of *Hypogymnia physodes*, provided by the author).

of environments, are mostly perennial, and their morphology does not vary with seasons so that the accumulation is continuous throughout the whole year (Szczepaniak and Biziuk, 2003). In addition, bryophytes and lichens lack a well developed root system, they have no cuticle (or it is very thin), and their leaves are one cell layer thick, so that they obtain nutrients over their entire surface directly from the atmosphere. These characteristics, together with the high surface to volume ratio and high cation exchange capacity in the cell walls of lichens and mosses, mean that these plants are very efficient at retaining trace elements in their tissues (Richardson, 1995; Markert et al., 1999).

Some authors have made direct comparisons of the feasibility of mosses, lichens and vascular plants (leaves and/or bark) for biomonitoring atmospheric heavy metal deposition. In the case of the comparisons between moss and tree leaves/needles, Ceburnis and Steinnes (2000) found concentrations of As, Cd, Cr, Pb and V that were several times higher in mosses (*Hylocomium splendens* (Hedw.) Schimp., *Pleurozium schreberi* (Wild. Ex Brid.) Mitt. and *Eurhynchium angustirete* (Broth.) T.J. Kop.) than in juniper

and spruce needles collected in the same sampling sites. These authors also examined the correlations between the concentrations of elements in mosses (assumed to be good biomonitors of atmospheric deposition of trace elements) and spruce needles, and concluded that the lack of correlation indicated that spruce needles could not be considered reliable biomonitors of air pollution. These results are similar to those reported by Aboal et al. (2004) in a study comparing oak leaves, pine needles and the terrestrial mosses *Pseudoscleropodium purum* (Hedw.) M Fleisch and *Hypnum cupressiforme* (Hedw.), and who concluded that the presence of a cuticle and epidermal cells in the leaves/needles of higher plants makes them less permeable than mosses to air pollutants, and that they would therefore be less reliable biomonitors of air pollution.

The results reported by Türkan et al. (1995) for a comparison between moss and tree bark were similar to the aforementioned findings for tree leaves/needles. These authors observed that the concentrations of Cd, Cr, Fe, Mn, Pb and Zn were between 3 and 9 times higher in *H. cupressiforme* than in pine bark in the surroundings of an iron-steel mill. Hence, they concluded that moss was a better accumulator of heavy metals and therefore more suitable for evaluating the extent of atmospheric pollution. These results are in direct contrast to those reported by Samecka-Cymerman et al. (2006), who found that bark was a better accumulator of Cd, Cu and Ni than the terrestrial moss *P. schreberi*. Nevertheless, the capacity of bark to accumulate trace elements is known to be determined by its chemical composition, and several studies have demonstrated that this is specific to each tree species and can be affected by tree age, health status, nature of the substrate in which the tree is growing (Bargagli, 1998), stand throughfall, stem flow (Hoodaji et al., 2012) and presence of epiphytic organisms (Bellis et al., 2001). Tree bark is also shaded by the tree canopy, which may intercept some of the airborne pollutants that would otherwise reach the bark. Therefore, on the basis of the aforementioned results and on morphological and physiological characteristics, mosses and lichens seem to be more suitable as than higher plants. Finally, despite having many features in common (see previous paragraphs),

the comparisons between mosses and lichens demonstrate that these plants are not interchangeable for monitoring atmospheric pollution because of differences in their morphology and ecophysiology (Bargagli et al., 2002). Thus, Salemaa et al. (2004) found higher concentrations of Cd, Cu, Fe, Ni, Pb and Zn in mosses than in lichens and vascular plants collected in the same plots along a pollution gradient. Furthermore, the differences were even greater in the most polluted sites. Coskun et al. (2009) found similar results in a comparison between epigeic *H. cupressiforme* and epigeic *Cladonia rangiformis* (Hoffm.), for As, Cd, Cu, Mn, Pb and Zn. Some researchers have also compared the levels of trace elements in moss and lichen transplants and found higher concentrations in mosses than in lichens (Giordano et al., 2005; Basile et al., 2008). Moreover, as identification and collection of lichens can be very complicated (Hoodaji et al., 2012) and they provide less biomass than mosses, the latter appear preferable for biomonitoring studies.

1.5 Mosses as biomonitors of atmospheric heavy metals deposition

1.5.1 Life cycle

Although some of the characteristics that make mosses good candidates for monitoring air quality have already been pointed out, a more detailed description of their life cycle and morphological and physiological features is given here. Mosses are the only member of the division Bryophyta and together with liverworts and hornworts constitute the phylum Bryophytes, the only land plants with a dominant gametophyte generation (Shaw and Renzaglia, 2004). The haploid gametophyte of mosses is long-lived and the most photosynthetically active generation in the life cycle (Fig. 3), while the diploid sporophyte generation is always short lived and produces haploid spores that germinate and grow into the gametophyte. The gametophyte generation has two distinct phases with very different life forms, the protonema stage and the leafy plant. The protonema consists of a green filament that grows immediately after spore germination and produces buds that grow into the mature gametophyte (leafy plant). Gametophytes may

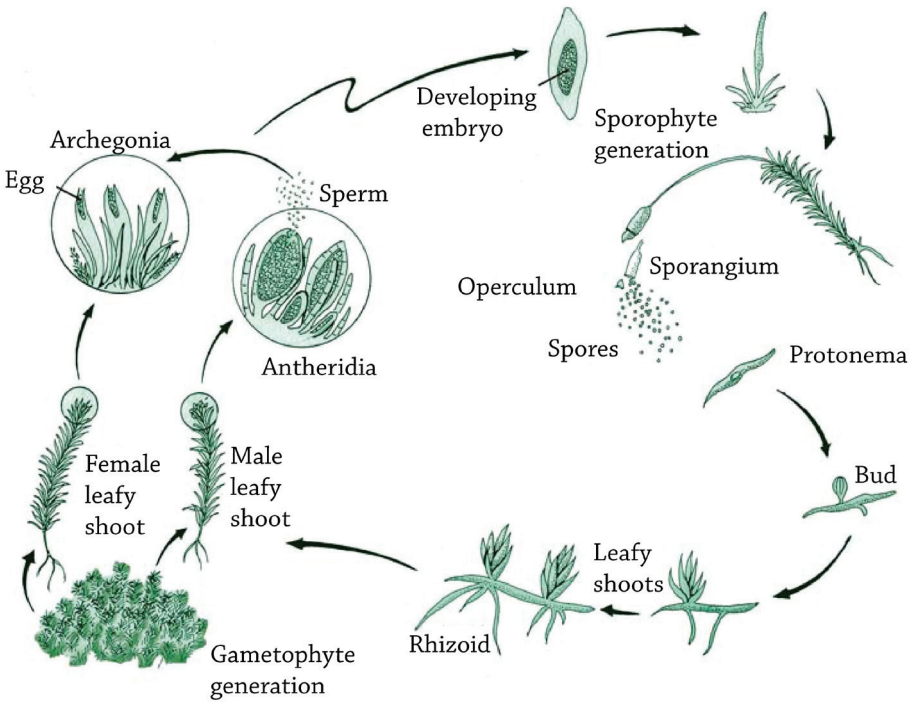


Figure 3: Life cycle of a typical moss (obtained from www.exploringnature.org).

be male or female (in the case of dioeicous species) or may have both sexes in the same plant (in the case of monoeicous species), and when sexual reproduction is about to occur, sperm and eggs are produced in structures called antheridia and archegonia respectively, which are usually located in the main stem. The sperm needs water to travel to the egg cells and once fertilization occurs, the zygote undergoes mitosis and becomes a multi-cellular sporophyte. Although sporophytes contain some photosynthetically active tissues, they mainly obtain their nourishment from the gametophyte. After production of spores, the sporophyte dies (Glime, 2013a).

Most moss species can also reproduce asexually, by producing asexual diaspores (specialized structures like gemmae or tubers), by regenerating from gametophyte fragments or even single leaves, or by clonal reproduction (separation of a branch from the parental plant giving place of a new ramet = daughter plant). This implies the rapid generation of a new gametophytic

plant, in which the more fragile protonema stage does not occur (a possible advantage in non-favourable environments).

1.5.2 Gametophyte traits

Taking into account that the gametophyte is the dominant generation in the moss life cycle, the characteristics of this stage will determine the suitability of the moss as a biomonitor. The gametophyte of mosses is composed of rhizoids (with some exceptions), stem, branches and leaves (Fig. 4B). Rhizoids mainly function by anchoring the plant to the substrate (they are not generally involved in uptake of water or nutrients), and they have a very simple architecture (each rhizoid is formed by a uniseriate filament of elongated cells) (Goffinet and Shaw, 2000). The stem anatomy is quite simple as it consists of a unistratose epidermis that always lack stomata, and the body is formed by non photosynthetic parenchyma cells. It can also contain more or less developed conducting systems, which consist in undifferentiated parenchyma cells, or specialized conducting cells (called hydroids), depending on the species, but never as well developed as in vascular plants. The leaves

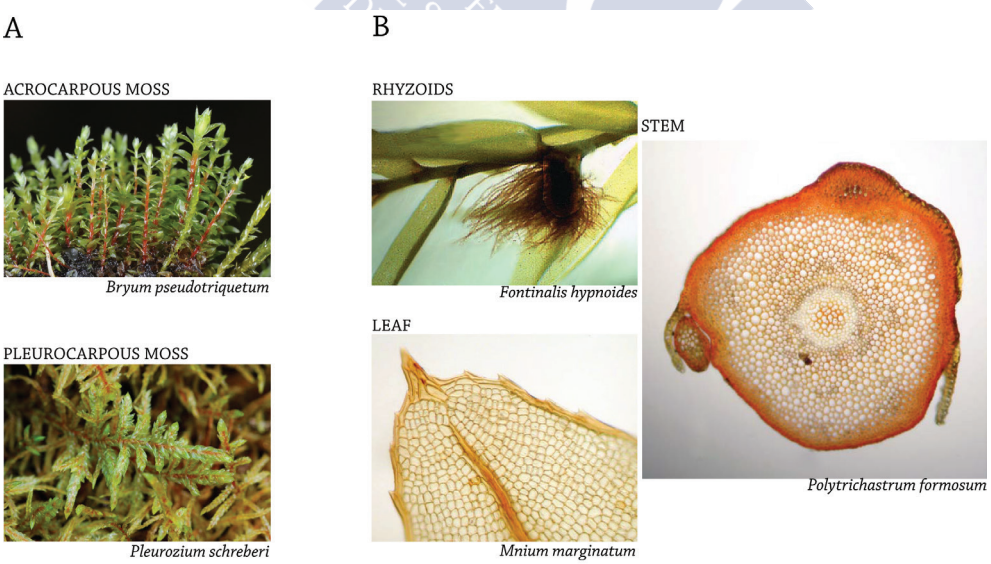


Figure 4: Morphological structure of moss gametophyte. A) Moss growth forms: acrocarpous and pleurocarpous; B) Detailed images of basic moss structures, rhizoids, stem and leaves (images obtained from www.biopix.es (A) and www.bryoecol.mtu.edu (B)).

of mosses are always sessile, i.e. they lack a petiole and their lamina is directly inserted on the stem. They are usually evenly distributed along the stem. The blade of a typical moss leaf comprises a single layer of cells, except in the median axis, which often forms a multistratose midrib (also known as costa). The leaves lack stomata and the cuticle is often reduced or even lacking (Vanderpoorten and Goffinet, 2009). Regarding the branching pattern, mosses can be classified as acrocarpous or pleurocarpous (Fig. 4A). The former are upright mosses in which sporophytes are located in a terminal position; they are usually unbranched or sparsely branched and grow vertically. The latter have the sporophytes in specialized lateral branches, and they are usually prostrate forming freely branched mats; they grow horizontally (Glime, 2013c).

All of the above-mentioned characteristics show that moss gametophytes are in very close contact with their surrounding environment and depend on it for their vital functions. In fact, these organisms are poikilohydric, i.e. the state of hydration is controlled by external water supply (Glime, 2013b), the primary source of which is the atmosphere. Nutrient uptake is also assumed to rely largely on minerals dissolved in rainwater and aerosols (wet deposition), as well as gases and dust (dry deposition). Morphological differences in moss gametophyte structure may play an important role in the nutrient uptake capacities of different species (Vanderpoorten and Goffinet, 2009). This also depends on the cation exchange capacity of mosses, which reflects the ability of the cell walls to bind cations due to the negative net charge that they possess (due to the presence of methylated uronic acids in the cell wall and outer layer of the plasma membrane: Richter and Dainty, 1989). Despite the simplicity of moss morphology and chemical structure, these organisms are very efficient at retaining both water and nutrients. As already mentioned, heavy metals may reach mosses together with nutrients via atmospheric deposition. The soluble forms of these elements are mainly cations (Cd^{+2}, Cu^{+2}, Hg^{+2}, Pb^{+2}, etc.), and mosses are therefore very efficient at taking up and retaining heavy metals. This constitutes the basis of the use of terrestrial mosses to monitor atmospheric heavy metal deposition.

1.6 Application of the moss technique to real studies: the beginning

The use of terrestrial mosses as biomonitors of air quality began in the late 1960s with the pioneering studies of Rhüling and Tyler (1968, 1970). These Swedish ecologists used the terrestrial mosses *H. splendens*, *P. schreberi* and *H. cupressiforme* to measure regional and historical trends of Pb deposition across Sweden (Rhüling and Tyler, 1968). They found differences in the levels of Pb between the northern and southern parts of the country and hypothesized that the higher concentrations were probably due to the proximity of the southern areas to the large industrial regions in Europe. Since then, the use of moss biomonitoring has been repeated in Sweden every five years and has been extended to other regions in Europe. Other Nordic countries (Norway, Finland and Denmark) were the first to join the programme in 1985 (Rühling et al., 1987), within the European Heavy Metal Deposition Programme established by the Environmental Monitoring and Data Group in the Nordic Countries. In 1987, the International Cooperative Program on Effects of Air Pollution on Natural Vegetation and Crops (ICP Vegetation) was established with the aim of obtaining monitoring data on heavy metal concentrations in mosses, among other aims. By 1990, 21 European countries had joined this programme; currently, 42 countries and more than 200 scientists from Europe and other regions in the world (i.e. Egypt, India, Japan, South Africa, USA) form part of the programme (Harmens et al., 2014). Monitoring of the spatial and temporal trends of atmospheric heavy metal deposition in Europe has therefore been undertaken for a long time.

Parallel to the development of national monitoring networks, biomonitoring studies using terrestrial mosses have been carried out at more local scales (e.g. the surroundings of particular industrial facilities), in order to describe the extent and the intensity of pollution in these environments, as well as the small scale patterns generated (e.g. Martin and Coughtrey, 1975; Glooschenko and De Benedetti, 1983; Brumelis et al., 1999; Uyar et al., 2008). However, the researchers' experience and observations made throughout these years of applying the moss technique, both at national

and local levels, have shown that the concentrations of heavy metals in moss tissues may be influenced by factors other than their atmospheric inputs. Hence, the dose-response relationship between moss concentrations and bulk deposition might also be altered. According to Markert et al. (2003), organisms that enable some conclusions to be reached about the concentrations of elements in the environment are those that maintain a linear relationship between the concentrations in their organism and the concentrations to which they are exposed (Fig. 5). If this relationship is altered, the conclusions that can be reached from biomonitoring studies may not be completely reliable.

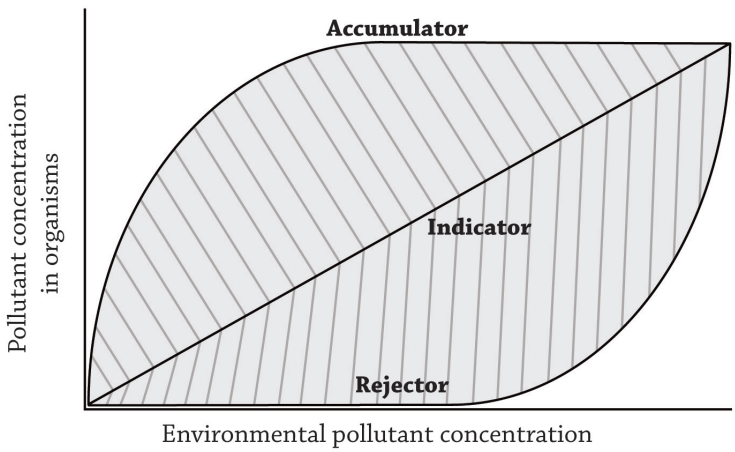


Figure 5: Classification of organisms according to the relationship between the concentration of the pollutant to be monitored in the environment and in the organism tissues (modified from Markert et al., 1997).

1.7 Factors influencing the results of biomonitoring studies

The factors that determine the final concentrations of elements in moss tissues in passive biomonitoring studies can be divided in two main groups: "avoidable" and "unavoidable" factors.

1.7.1 "Unavoidable" factors

Unavoidable factors are processes or characteristics intrinsic to the organ-

ism and to the technique and the effects of which cannot be controlled or avoided. Such factors alter the possible linear relationship between the concentrations of elements in moss and in the atmosphere, thus limiting the conclusions that can be reached. These factors include, above all, the presence of sources of pollutants other than atmospheric deposition. Indigenous mosses are directly exposed to soil dust, sea spray aerosols and transfer of elements from higher plants (by leaching from living and dead plant tissues). These are well known additional sources of some elements to mosses (i.e. Cr and Fe are common in soil dust; Mn and Rb are commonly leached from higher vegetation, etc.) (Steinnes et al., 1992; Berg et al., 1995). Secondly, the physicochemical characteristics of pollutants are also important. The form in which the pollutant is emitted (i.e. gaseous, particulate, ionic) will determine the way in which it is deposited (dry/wet deposition) and also the way in which it will be taken up and retained by mosses (e.g. in cation exchange sites in the case of ionic forms, trapped on moss surface in the case of particulate matter) (Fernández et al., 2004). This will also determine how the pollutant interacts with other elements present in the atmospherically deposited material (cation competition, anion complexation, etc), thus enabling or preventing its uptake by mosses.

Thirdly, the moss biology is also important because, despite being quite simple organisms, mosses have physiological needs and the uptake of at least some essential nutrients (i.e. Cu, Cr, Zn) might be more or less regulated. The morphology and chemical composition of mosses will also determine their ability to take up and retain pollutants, and moss growth will determine both the time of exposure of tissues to atmospheric deposition and their metabolic activity. All of these characteristics related to moss biology may vary between species, but also within the same species, as they largely depend on environmental and microenvironmental factors (i.e. temperature, humidity, radiation, etc.) (Zechmeister, 1995). Finally, environmental factors are also important. Precipitation may remove particles attached to moss by mechanical action or solubilise elements already attached to particles. On the other hand, the lack of precipitation will lead to dehydration of

tissues, thus favouring the release of particles (Fernández et al., 2010).
As already pointed out, the effects of these processes cannot be avoided in passive biomonitoring; however, having information about the processes would help improve interpretation of the results or highlight the need for caution in several aspects. Unfortunately, very little is known about the way that many of these factors (i.e. mechanisms of metal uptake, effect of moss growth on the concentrations found, effect of these elements on mosses and possible development of adaptations, etc.) affect the concentrations found in mosses, and further studies of these aspects are required. Hence, the first part of this doctoral thesis comprises a detailed study of the effect that some of the above-mentioned processes have on the concentrations of trace elements in moss tissues, and the implications of the effects. The species of moss selected for all the experiments was *Pseudoscleropodium purum* (Hedw.) M. Fleisch., as this species is widely distributed in the northern hemisphere and, more specifically, in Galicia (NW Spain) (where all the experiments were carried out). Moreover, the Ecotoxicology research group of the University of Santiago de Compostela, with which this research was carried out, has more than 20 years of experience in passive and active biomonitoring studies with this and other species.
Some authors have stated that the moss technique provides a time-integrated measure of atmospheric deposition of heavy metals (Rühling, 1994; Čeburnis and Valiulis, 1999; Schröder et al., 2013). Thus, the portion of the moss shoot selected for the analysis should give information about the deposition received during the time that these tissues had been exposed. Therefore, the only factor that would affect the concentrations over the time would be the biomass increment, that is, moss growth. However, it is known that many other factors affect the concentrations of heavy metals in moss tissues and that these vary over time (including moss growth) and their effects will therefore not always be the same. Moreover, very few studies have addressed the effect of moss growth on the concentrations of elements in mosses. Therefore, Chapters I and II of this thesis will assess the temporal variability of the concentrations of several nutrients and pollutants in *P.*

purum sampled with high frequency and the effect of growth on the concentrations of elements in segments of different ages of *P. purum*, respectively.
Secondly, most researchers who have carried out studies of passive biomonitoring have not generally considered the possibility that mosses may become adapted to the severe conditions under which they live in polluted environments. Taking into account that pollutants affect moss physiology/metabolism (Brown and Wells, 1990; Choudhury and Panda, 2005), and that this may have consequences for moss fitness, it appears logical that mosses may have undergone adaptations to deal with atmospheric pollution. Therefore, Chapter III of this thesis examines, by means of a cross-transplant experiment, whether mosses that have grown in environments under different levels of pollution show different accumulation capacities.
Finally, some authors have tried to use the moss technique to obtain absolute heavy metal deposition values (Čeburnis et al., 1999; Čeburnis and Valiulis, 1999). This could be done by converting moss concentrations into deposition rates, by calibrating moss data against bulk deposition values. However, the studies in which this type of calibration has been carried out (i.e. Ross., 1990; Berg et al., 1995; Thöni et al., 1996; Berg and Steinnes, 1997; Schintu et al., 2005, and Fowler et al, 2006) have shown that the correlations between moss and bulk deposition data vary between species (for the same elements), between sampling sites (for the same species) and between elements (for the same moss species). Therefore, it is still not known whether these calculations can be done or even whether moss concentrations and bulk deposition levels of heavy metals are correlated. Thus, Chapter IV consists of a critical literature review in which the results of diverse studies serve to illustrate why the conversion of concentrations of elements in moss into heavy metal deposition rates is not reliable. In addition, Chapter V discusses the specific case of Mn, an element for which significant correlations between moss concentrations and bulk deposition have never been shown, and which has already been proposed for exclusion from regional surveys (Ross, 1990; Ceburnis et al., 1999). Therefore, the aim of this chapter is to study the processes that affect Mn bioconcentration by mosses

in depth and to decide whether or not this element should be excluded from regional surveys. Finally, Chapter VI reports a detailed study of the relationship between the concentrations of several heavy metals in mosses and in bulk deposition.

1.7.2 "Avoidable" factors

Avoidable factors are those factors related to the experimental set up of biomonitoring studies and that affect the comparability of the results of different studies. Where, when and how moss samples are collected (experimental design) will affect the outcome of this type of study (Wolterbeek and Bode, 1995), and hence, the degree to which the final objective is fulfilled. This is especially true for extensive biomonitoring networks such as those established within the programme of the ICP Vegetation, because differences in sample collection, processing and chemical analysis will hamper the comparability of the results obtained. Aspects such as the moss species selected, the density of sampling sites (SS), the spatial arrangement of SS, area of the SS, size of sample collected, part of moss shoots selected for the analysis, etc. clearly influence the results. As mentioned above, the effect of these factors could be avoided, or at least minimised, by establishing a harmonized biomonitoring protocol for use in the monitoring programs carried out worldwide.

Until now, and after more than 50 years working in the field of passive biomonitoring of air quality using terrestrial mosses, the only existing manual for sampling mosses in national surveys is that published by the ICP Vegetation (the last manual was the published for the 2015 survey – Frontasyeva and Harmens, 2014). This guideline presents a series of recommendations regarding the methodological aspects involved in passive biomonitoring studies using terrestrial mosses. However, some of these recommendations are based on scientific criteria and others are not. Therefore, Chapter VII of this thesis consists of a critical review of the available literature on passive biomonitoring with terrestrial mosses in order to propose a protocol, as harmonised as possible, based on the results of the methodological studies

published to date. Thus, each step involved in the development of this type of study will be described and discussed in detail.

1.8 State of the art

The main problem when establishing a reference protocol for passive biomonitoring with terrestrial mosses is that the number of studies addressing methodological aspects is very limited. These studies are essential in order to improve the quality and the comparability of the results obtained and to identify the limitations of the technique. However, of the 369 relevant articles published between 1972 and 2014, 68% focused on biomonitoring of air pollution at different sites (Fig. 6). Therefore, only a small proportion of papers address methodological aspects. The best studied aspect in these type of study is the effect of soil or substrate in moss concentrations (6% of the studies), i.e. the contribution of this source of elements to the final results.

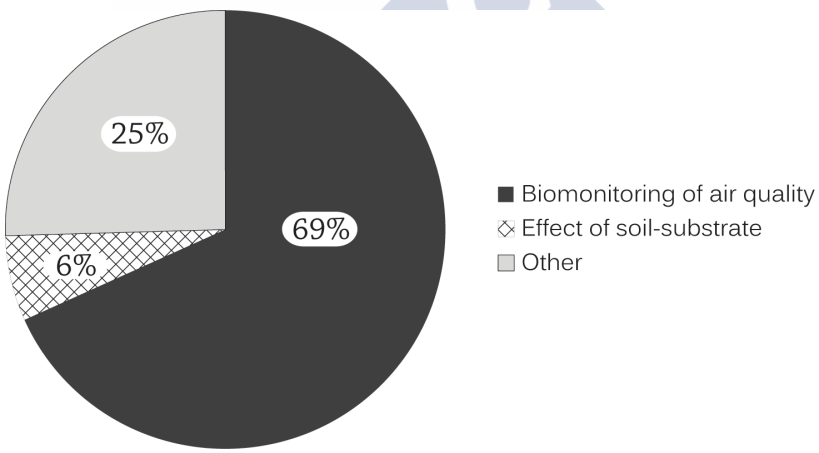


Figure 6: Percentage of studies (from a total of 369 published works) using the passive biomonitoring technique with terrestrial mosses to monitor atmospheric deposition of trace elements compared with the percentage of articles addressing methodological aspects. "Others" includes studies addressing aspects such as sampling design, and the effects of vegetation, altitude, species, growth, etc., on the concentrations of elements in moss tissues.

The remaining 25% of studies focused on studying several other aspects regarding the pre-sampling issues (i.e. sampling design - 2% of the studies - interspecies comparisons - 5% of the studies), sampling issues (i.e. effect

of vegetation (2%) and altitude (2%) in the concentrations found in moss tissues; representativeness of the sampling site (2%)), and post-sampling issues (i.e. analytical techniques – 3%). Furthermore, although the number of articles published annually on this topic increased considerably between 1995 and 2008 (Fig. 7), there remains some bias towards studies in which the simple application of the technique was carried out, which is probably the reason for the low degree of harmonization of the technique. Finally, the number of published papers about passive biomonitoring of trace element deposition with terrestrial mosses tended to decrease after 2008. Future literature reviews should elucidate whether this tendency is maintained over time, as well as the possible reasons for the decrease.

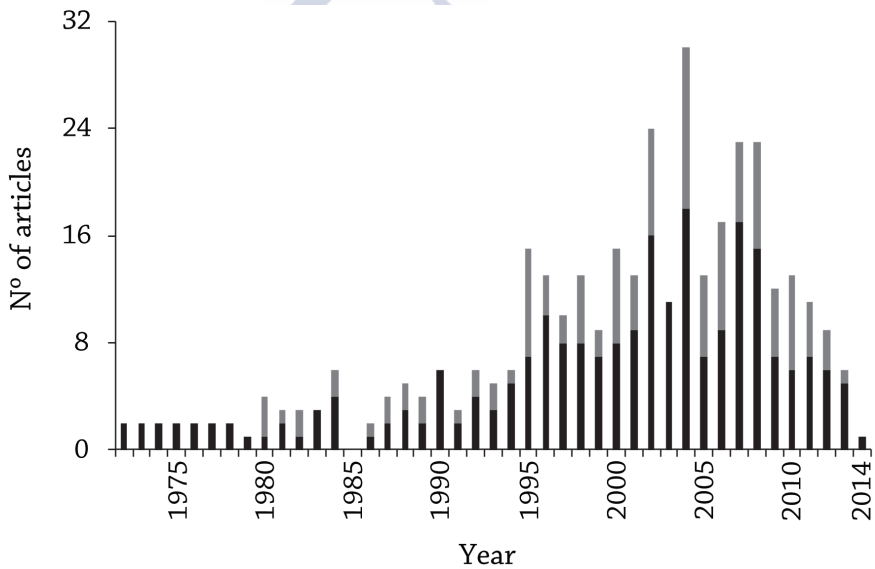


Figure 7: Number of articles involving passive biomonitoring with terrestrial mosses to monitor atmospheric deposition of trace elements (black bars) compared with the number of articles addressing methodological aspects (grey bars) per year between 1972 and 2014.

The lack of studies in which methodological aspects are assessed may be due to the lack of specialized researchers in this field. Thus, Figure 8 shows the number of papers written by each author; only 11 authors published more than 10 papers in the field, while most of the authors (in total 521) pub-

lished only one paper.

Although the moss technique has emerged as a valuable tool for monitoring the atmospheric deposition of heavy metals, it has also been used to monitor the deposition of persistent organic pollutants (POPs). The majority of stud-

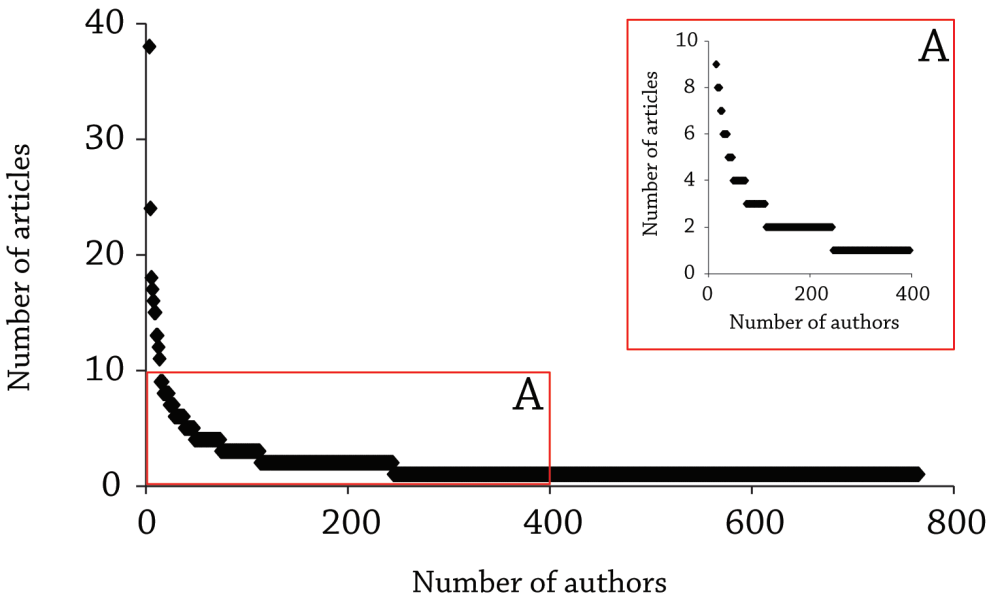


Figure 8: Number of articles published per author in the field of passive biomonitoring of atmospheric trace elements deposition with terrestrial mosses. Detailed view of the number of authors who published fewer than 10 articles on this topic (Inset A).

ies have focused on the determination of polycyclic aromatic hydrocarbons (PAHs), and very few have focused on other POPs such as organochlorines (OCs) or dioxins and furans (PCDD/Fs) (see more detailed information in Harmens et al., 2013). In the case of inorganic pollutants, under study in this thesis, a total of 89 different elements were determined in more than 350 studies. Of these, the elements most commonly considered were Pb, Zn, Cu, Cd, Ni, Fe and Cr (determined in more than 50% of the papers reviewed – Fig. 9A), probably because these are among the 10 heavy metals recommended by the ICP vegetation for consideration (shown in blue in Fig. 9A) (Harmens et al., 2004). Most plant macronutrients (shown in green in Fig. 9) are among the most commonly measured elements, as well as two

radioactive elements (Th and U). Elements considered in less than 10% of the studies ("least commonly determined elements" - Fig. 9B) include P and N (plant macronutrients) and some Pb isotopes.

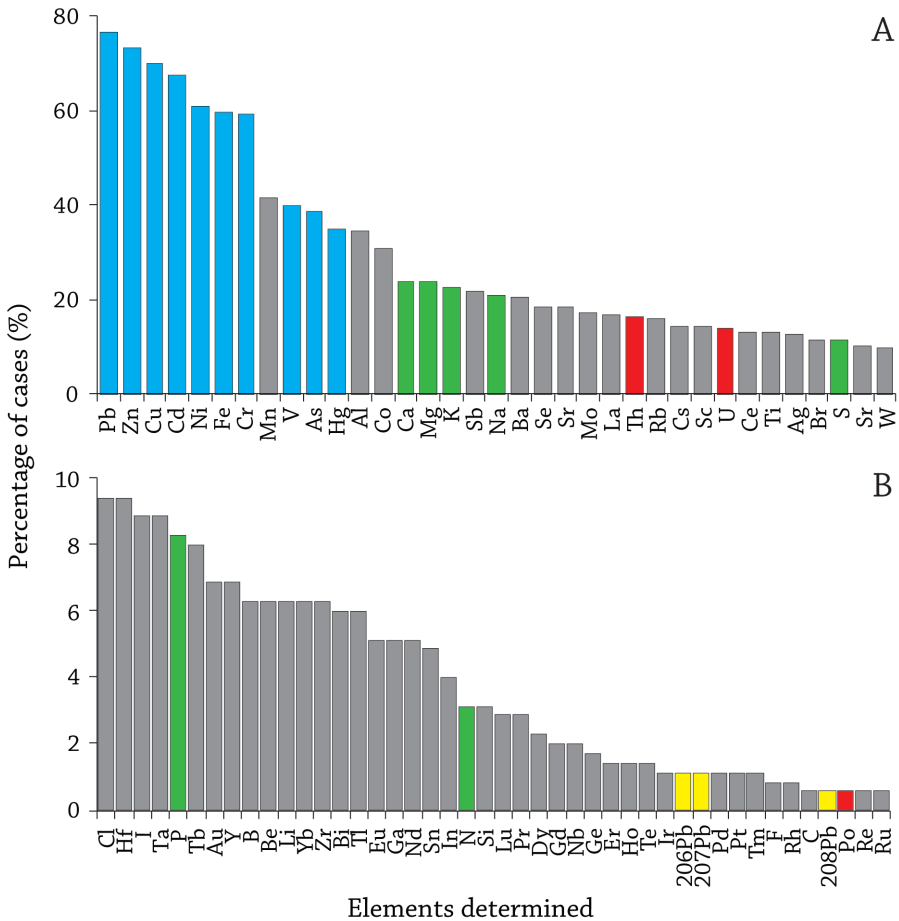


Figure 9: Frequency with which the different inorganic elements have been determined in a total of 369 studies. A) Elements most frequently determined (in more than 10% of cases); B) Less frequently determined elements (in less than 10% of cases). Blue bars: elements recommended for measurement by the ICP Vegetation; green bars: main plan macronutrients; red bars: radioactive elements; yellow bars: isotopes; grey bars: the rest of elements.

Finally, it can be observed from Figure 10 that the moss technique is mainly used in Europe (80% of the published works), more specifically in northern countries (41% of the works reporting studies within Europe) where the technique was first proposed. The other 59% European articles report

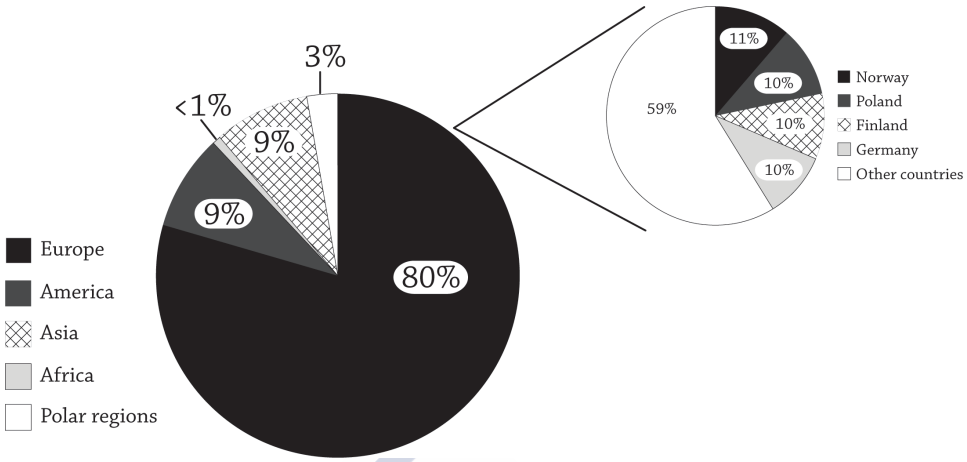


Figure 10: Percentage of studies (from a total of 369 published works) on the passive biomonitoring of atmospheric trace elements deposition with terrestrial mosses carried out worldwide (A). Detailed view of the percentage of articles published in the different European countries (B). "Other countries" includes a total of 27 different European countries.

studies carried out in 27 different countries. The only possible and objective explanation (given the state of the art of the technique) for the widespread use of this technique in Europe, but not worldwide, is that this began as a revolutionary tool, with all the possibilities and advantages previously mentioned in this section. However, until now it has never been regulated by the legislation (to our knowledge, the only country in which the technique was considered in the legislation is Finland), and therefore it has not been applied by governments in control and prevention policies and is mainly restricted to research circles. This is probably because the lack of standardization of the technique has made decision-makers sceptical about biomonitoring methodologies.

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GENERAL OBJECTIVES



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General Objectives

The main objectives of this thesis can be grouped in two parts. Thus, one part of the thesis will try to assess the reliability of the moss technique for monitoring the atmospheric deposition of heavy metals. For this purpose, the relationship between the concentrations of these elements in moss tissues and in bulk deposition will be studied in depth, and the factors affecting the concentrations in moss, and hence altering this relationship, will be assessed and discussed. The possible response of mosses to continuous exposure to high levels of atmospheric heavy metal deposition will be also studied. This information will be used to highlight the limitations of the technique, and possible solutions or alternative methods of interpreting the results of passive biomonitoring studies with terrestrial mosses will be proposed (Chapters I to VI).

The second part of the thesis will consist of the compilation of as much information as possible regarding use of the moss technique. A harmonised and scientifically based protocol will then be proposed for application. The main scope of this protocol will be to unify the information obtained from articles reporting methodological aspects to provide scientific support for all the decisions/steps involved in planning this type of study. Less well studied aspects will be identified and the need for further research on these topics will be highlighted. This will improve the quality and the comparability of the results of studies applying the moss technique (Chapter VII).

More specifically, the aims of the present thesis are as follows:

1. To assess the temporal representativeness of the concentrations of trace elements in terrestrial mosses and the implications that the temporal variability in the concentrations may have on the results of passive biomonitoring studies with these organisms. For this purpose, the concentrations of various pollutants and nutrients were measured in samples of *P. purum*, by high frequency sampling over a long period of time (Chapter I).

2. To study moss growth rates during several well defined periods, and to evaluate the effects of growth/tissue ageing on the concentrations of elements in mosses and the implications for the results of biomonitoring studies. For this purpose, annual growth rates of *P. purum* were measured in different environments, and within-site variability in moss growth was assessed. The temporal changes in the concentrations of various elements in moss segments of different ages were also studied (Chapter II).

3. To check for differences in the capacity of mosses growing naturally under different levels of pollutant inputs to accumulate heavy metals. Specimens of moss were cross-transplanted between two different polluted areas and an unpolluted area, and temporal changes in the concentrations of various heavy metals were measured (Chapter III).

4. To conduct a critical review of the studies published to date involving the estimation of atmospheric heavy metal deposition rates by means of moss data. The reasons why the results of these studies are not reliable, and the reasons why bulk deposition should not be estimated from the concentrations of elements in moss (based on the available literature) are discussed (Chapter IV).

5. To determine whether terrestrial mosses are good biomonitors of the atmospheric deposition of Mn, an element for which no significant correlations between concentrations in moss and bulk deposition have ever been observed (Chapter V).

6. To study in depth the relationship between the concentrations of several heavy metals in mosses and in bulk deposition. The experimental set up of this work was carefully designed in order to enable reliable conclusions to be reached about this relationship, and some improvements were made with respect to previous studies in this field (Chapter VI).

7. To propose a harmonised protocol for the application of the passive

biomonitoring of air quality with terrestrial mosses. For this purpose, a total of 369 papers published between 1972 and 2014 were revised (Chapter VII).

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CHAPTER I

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Analysis of temporal variability in the concentrations of some elements in the terrestrial moss *Pseudoscleropodium purum*

Environmental and Experimental Botany, 2011, 72: 210-216.

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Abstract

The uptake of contaminants by moss is a complex process that depends on the nature and characteristics of the contaminants and on the physicochemical and biological processes that affect the moss. All of these factors in turn depend on environmental variables that may vary within a short time. It can therefore be assumed that the uptake of elements varies in such short periods that it is very difficult to obtain temporally representative results in biomonitoring studies with terrestrial moss. In order to investigate the temporal variability and the implications that it may have on the interpretation of the results obtained in this type of study, the tissue concentrations of twelve elements were determined in samples of the moss *Pseudoscleropodium purum* collected from two sampling sites in Galicia (NW Spain), during a long term survey with a high frequency of sampling. The results showed that the concentrations of the elements in the moss varied greatly within very short periods, and that the error associated with the temporal variability in the results was high. In accordance with these findings, and taking into account that the technique enabled contaminated and uncontaminated sites to be distinguished, we recommend that the results of biomonitoring studies should be regarded as qualitative or semiqualitative, rather than attempting to provide absolute data, which may not be temporally representative, and may have a high degree of uncertainty associated with them.

Keywords: *terrestrial bryophytes, biomonitoring, atmospheric contamination.*

1. Introduction

Since they were first used in the late 1960s (Rühling and Tyler, 1968, 1970), terrestrial mosses have become one of the most commonly used

tools for biomonitoring the air quality worldwide. The extensive use of the moss monitoring technique is largely due to the fact that the technique is inexpensive and easy to apply. However, despite its widespread use, certain aspects of the technique must be improved for better interpretation of the results obtained. One such aspect is related to the spatial and temporal representativeness of the data obtained from moss samples.

Although the spatial representativeness of the results has been analysed in several studies (Aboal et al., 2006a; Boquete et al., in press; Fernández et al., 2005; Figueira et al., 2007, Schröder et al., 2004, Wolterbeek and Verburg, 2004), to date, little attention has been given to the temporal representativeness of the results. For example, it is not known what time period is reflected by the concentration of a metal in a moss sample collected at a particular moment.

For an organism to qualify as a good biomonitoring agent, it should be capable of integrating the contaminants that it receives, so that there is a direct correlation between the concentration of an element to which the organism exposed and the concentration of the element in the tissues (Phillips, 1980). It was originally though that terrestrial mosses integrate atmospheric contamination, so that the concentrations of the different elements in moss would mainly depend on their complete precipitation (see for example: Ross, 1990; Berg et al., 1995; Thöni et al., 1996; Berg and Steinnes, 1997; Schintu et al., 2005). Any variations would therefore be due to changes in rates of growth of the organism, in addition to the variations in inputs of contaminants. However, more recently, some authors have reached the conclusion that mosses do not integrate atmospheric contamination, but that they are in equilibrium with the environment (Couto et al., 2004). This is a complex equilibrium that depends on various factors. Furthermore, it must be remembered that the atmosphere is not the only source of contaminants for moss, as in some cases both the soil and vegetation supply considerable amounts of certain elements (see for example: Steinnes, 1995; Berg et al., 1996; Fernández and Carballeira, 2001). In addition, these inputs will differ depending on the nature of the contaminant and the form in which it is emitted. On one hand, the inputs and outputs of element in moss will depend on physicochemical

processes (i.e. solubilization and leaching of elements, cation competition, anionic complexation etc.) and biological processes (rate and type of growth, physiological activity, phenotypic adaptations, etc.). Finally, all of these variables will depend on environmental factors (precipitation, pH, salinity, temperature, etc.), which in turn may vary within short periods of time.

Taking all of this into account, it can be concluded that the uptake of elements by moss is a very complex process that may vary on a very short timescale. However, to date, a low frequency of sampling (the shortest interval has been 1 month) has been used in most studies involving temporal analysis of the concentrations of contaminants in terrestrial moss (Markert and Weckert, 1989, 1993; Thöni et al., 1996; Faus-Kessler et al., 1999; Fernández and Carballeira, 2002; Couto et al., 2003, 2004; Zechmeister et al., 2003; Leblond et al., 2004); only one of these studies explored the temporal representativeness of the results (Couto et al., 2003). Despite this, in some of these studies, large temporal variations in the concentrations of contaminants were observed. For example, Markert and Weckert (1989) observed seasonal variations in the concentrations of up to 50% for some elements and in some cases, up to 80%. Likewise, Real et al. (2008) recently reported differences in concentrations of Hg ranging from approximately 500 ng·g-1 to 3200 ng·g-1 in an interval of one month, in a study in which sampling was carried out monthly or every two months for different lengths of time between 1996 and 2005.

Although temporal variations in the concentrations of various elements have been observed in some terrestrial mosses, these have generally been overlooked and the possible implications for interpretation of the results of biomonitoring studies have not been considered. The aim of the present study was therefore to analyse the temporal variability in the concentrations of diverse contaminants and nutrients in the terrestrial moss *Pseudoscleropodium purum*, by high frequency sampling over a long period. The implications that the variability may have for interpretation of the results obtained in moss biomonitoring studies are also discussed.

2. Material and methods

2.1. Sampling

Samples of the terrestrial moss *Pseudoscleropodium purum* (Hedw.) M. Fleisch. were collected from two sampling sites (SS) in Galicia (NW Spain). At both sites, the samples were collected twice weekly (i.e. every 3 or 4 days), during the entire sampling period. At the first site (SS1), located in the surroundings of an aluminium smelter and that contaminates the area with various heavy metals and metalloids (Fernández et al., 2007), the sampling began on 3 January 2005 and continued until 29 December 2005 (n = 101). At the second site (SS2), corresponding to an uncontaminated area (Aboal et al., 2004), the sampling began on 29 May 2005 and ended on 29 December 2005 (n = 62).
The samples were collected from open areas; in each SS more than 30 subsamples of similar weight and distributed uniformly throughout areas of approximately 30 x 30 m, were collected (Fernández et al., 2002, Aboal et al., 2006b, Varela et al., submitted).

2.2. Processing and chemical analysis

Prior to their analysis, the samples were cleaned, and apical segments (3-4 cm length) were cut from the shoots; all material adhering to the segments was removed. Half of each sample was maintained in a moisture-saturated atmosphere at 10ºC for one week to ensure the integrity of the membranes. This part of the sample was then washed with bidistilled water, with shaking for 30 sec. Finally, both the washed and unwashed samples were homogenised in an ultracentrifugal mill (Retsch ZM100) then digested with HNO_3 (65%, analytical grade) in a microwave oven (CEM MDS2100).
The concentrations of Al, Cd, Cr, Cu, Fe, K, Na, Ni, Pb, V and Zn in the samples were measured by flame atomic absorption spectrometry (Perkin Elmer 2100) or, when the concentrations did not reach the limits of quantification (Ni in samples from site SS2, and Cd, Pb and V in both SS), by graphite chamber atomic adsorption spectrometry (Perkin Elmer AAnalyst 600). The concentrations of Hg were determined in an element analyser (Milestone DMA80).

To ensure the analytical quality of the determination of Hg, two replicates of each sample were analysed; for the other elements, two replicates of the same sample were analysed every nine samples. In addition, four certified reference materials were analysed: Poplar (GBW07604 poplar leaves - Institute of Geophysical and Geochemical Exploration, Langfang, China), M2 and M3 (*Pleurozium schreberi*, Steinnes et al., 1997) and an internal reference material (*Pseudoscleropodium purum*), also once every nine samples. To control for any possible contamination, analytical blanks were also included, once every nine samples. In general, the analytical quality of the process was satisfactory, with some exceptions the overall percentage of error (Cêburnis and Steinnes, 2000) was less than 10% (between 4 and 5% for Cu, Hg, Na and Zn), and the percentage recovery was close to 100%. The error was higher than 10% and the percentage recovery far from 100% for only a few elements (i.e. Al, Cr, Fe and Ni), the latter because of the siliceous matrix; these elements are mainly deposited in particulate form or are present in edaphic material, and therefore the samples are very heterogeneous.

2.3. Statistical analysis

The total percentage error associated with the temporal variability in the concentrations of some elements was calculated. Assuming that the error due to temporal variability and spatial variability at the sampling plot level are independent from each other, the total error corresponds to the sum of the squares of these sources of error. The following expression was used to calculate the total error:

$$E_{total} = \sqrt{E_M^{\,2} + E_{VT}^{\,2}}$$

where: E_{Total} is the total error associated with the determination of the concentrations of each element at each SS, and corresponds to the standard deviation of the concentrations. E_M is the error due to the spatial variability in the concentration in moss within the sampling plot, along with the error due to the analytical determination of the concentrations of various elements in

the moss. The latter (E_M) was obtained by calculation of the standard deviation of the concentrations of Cd, Cu, Fe, Hg, K, Ni and Zn in three SS in Galicia (NW Spain). These data, obtained in a study in which 50 subsamples of the moss *P. purum* were collected from each of three SS and analysed separately, have already been reported (Aboal et al., 2006b). Calculation of the standard deviation of the concentrations of each element in each of the plots therefore enabled estimation of the analytical and sampling error associated with measurement of the concentrations. Finally, E_{VT} is the error due to the temporal variability in the concentrations of elements in the moss tissues.

3. Results

The tissue concentrations of the different elements in the washed and unwashed moss samples are shown in Fig. 1 and Fig. 2 respectively. In addition, bars representing the analytical error associated with the measurement of each element and the moving mean (for n=5) of all of the concentrations of each element, are shown in the corresponding graphs. For both the washed moss and the unwashed moss, the concentrations of the contaminants fluctuated widely with time in both SS, which was apparent even by only studying the moving mean. This variation was particularly notable at the end of the year, so that the peak concentrations generally occurred in the last three months of sampling. However, the concentrations of Hg remained more or less constant throughout the sampling period, until the end of the year, when they increased sharply. The temporal patterns of variation of some of the other elements, such as Cr, Fe and Ni, were very similar. In addition, the concentrations of Na appeared to follow a seasonal pattern, with high concentrations at the beginning and end of the year, and lower concentrations in the summer period.

The percentage variations in the concentrations of the elements were calculated for different periods. For this purpose, pairs of data obtained 3-4, 6-8, 9-12 and 12-16 days apart were obtained and the absolute value of the difference in concentration was, in each case, divided by the initial and final concentration. The mean percentage variations for each element, SS and

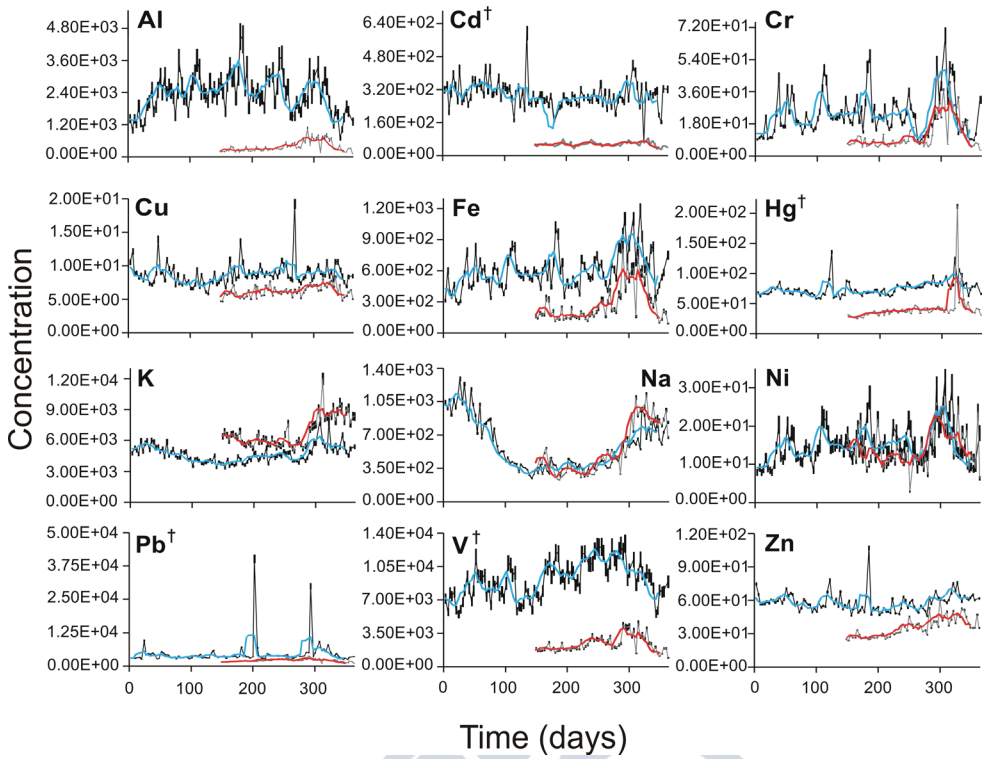


Figure 1: Differences in the concentrations (Y axis) (μg·g^{-1}, †: ng·g^{-1}) of different elements in the washed moss *Pseudoscleropodium purum* over time (X axis), in samples collected every 3-4 days during the entire sampling period. The bars represent the error associated with the analytical determination of the elements. The black line represents the concentrations of the elements in the contaminated SS (SS1) and the grey line, the concentrations in the uncontaminated site (SS2). The moving mean is also shown for each series of data.

period are shown in Table 1, along with the upper and lower limits of the range in variation. For some elements, the range of variation in the concentrations was very large, i.e. for Hg and a period of two weeks, the range of variation was between 1% and 529%; for Cd and samples collected 3-4 days apart, the range of variation was between 2% and 520%. According to these data, the variation in the concentrations of some elements in samples separated by up to 15 days may be extremely high.

In addition, for each element and SS, the coefficient of variation and the 1% and 99% percentiles of the concentrations obtained throughout the entire sampling period were calculated (Table 2). For elements such as Al, Fe, Hg

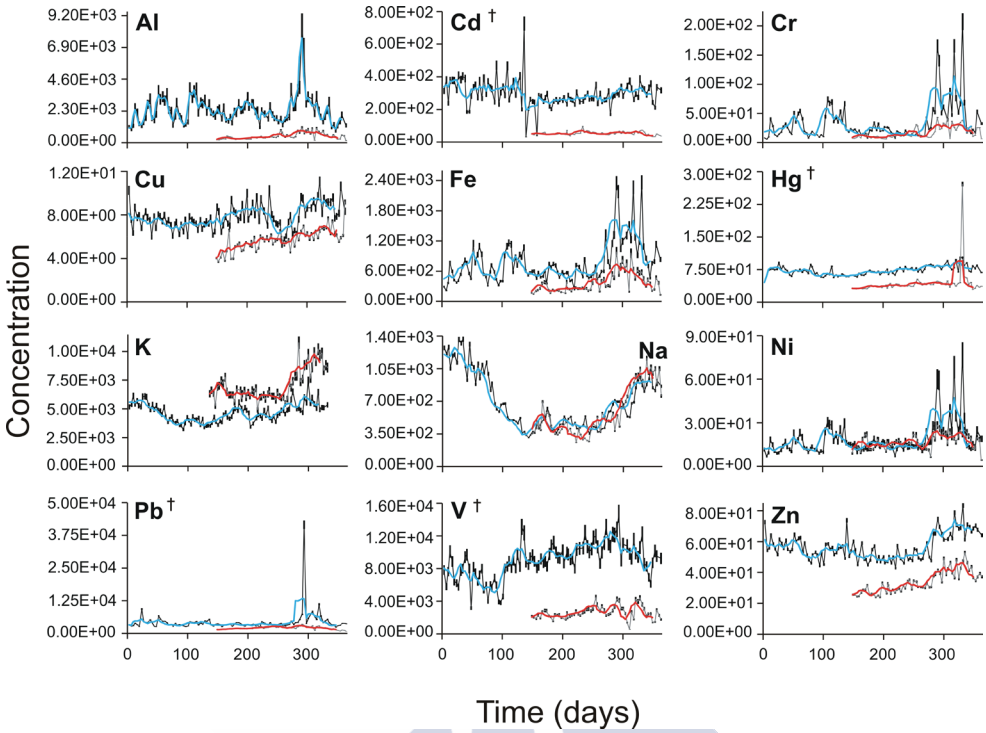


Figure 2: Differences in the concentrations (Y axis) ($\mu g \cdot g^{-1}$, †: $ng \cdot g^{-1}$) of different elements in the unwashed moss *Pseudoscleropodium purum* over time (X axis), in samples collected every 3-4 days during the entire sampling period. The bars represent the error associated with the analytical determination of the elements. The black line represents the concentrations of the elements in the contaminated SS (SS1) and the grey line the concentrations in the uncontaminated site. The moving mean is also shown for each series of data.

and Ni, the coefficient of variation was higher than 50% in some of the SS, and reached up to 99% in the case of Cr in SS1. Both the percentages of variation in the concentrations at different intervals and the values of the percentiles show that the range of temporal variation in the concentrations of the different elements was very high in both the washed and unwashed moss samples.

As regards the differences between the two SS, the concentrations of almost all of the elements were higher in the contaminated site (SS1), although the concentration of K, a nutrient, was higher in the samples from the uncontaminated site (SS2). This, combined with the fact that the analytical error associated with the determination of elements in terrestrial moss was very

low in most cases, meant that it was possible to differentiate the contaminated and uncontaminated zones in qualitative terms. Thus, the error bars show that the lower concentrations of contaminants in the contaminated site (SS1) were higher than the highest concentrations of contaminants in the uncontaminated zone (SS2), and vice versa for nutrients.

Finally, the total percentage error due to the temporal variability in the data was higher than 80% in most cases for Cu, Hg and K; for Fe, the error was greater than 90% and for Cd and Ni reached 100%. The percentage error was low -approximately 50%- only for Zn. These data show that the sampling error and analytical error are very low in comparison with the temporal error and therefore that the temporal variability in the data has a large effect on the final concentrations obtained.

4. Discussion

The concentration of different elements in moss is the final result of a complex equilibrium in which various factors interact. These factors ultimately depend on the environmental conditions, which in turn vary with time. Thus an important degree of variation is generated in the concentrations of elements in moss over time.

The results of the present study demonstrate a large degree of variation in the concentrations of all of the elements determined (contaminants and nutrients) in the moss tissue within short periods of time, independently of whether the samples were washed or not. Thus, for example, the concentrations of Al in samples of moss from contaminated SS varied by 1960 $\mu g \cdot g^{-1}$ in one week, and by 2700 $\mu g \cdot g^{-1}$ in two weeks. Furthermore, it was found that in SS1, the concentration of V ranged from approximately 6000 to 10000 $ng \cdot g^{-1}$ in only 3 days. Variations in the concentrations of Cd of up to 5 times were also observed within only 3 days in the contaminated SS. However, these variations in concentration did not only occur in the contaminants, and for example the concentrations of K in the uncontaminated SS ranged between 5000 and 12000 $\mu g \cdot g^{-1}$ throughout the sampling period, with variations of up to 3780 $\mu g \cdot g^{-1}$ within periods of 3 days. This trend was observed

Table 1: Mean difference in concentration (and range of variation) (%) between pairs of data separated by different intervals of time (3-4, 6-8, 9-12 and 12-16 days), for each element measured in the moss *Pseudoscleropodium purum* and for each sampling site (SS). B: difference in concentration between the pairs of samples (absolute value) divided by the value of the first concentration; A: difference in concentration between the pairs of samples (absolute value) divided by the value of the second concentration.

Interval of time (days)			Al	Cd	Cu	Cr	Fe	Hg	K	Na	Ni	Pb	V	Zn
3-4	SS1	B	20 (1-65)	16 (0-417)	11 (0-145)	26 (0-154)	16 (0-91)	7 (0-56)	8 (0-57)	14 (0-73)	16 (1-124)	15 (0-1058)	9 (0-45)	8 (0-68)
		A	21 (1-127)	16 (0-412)	11 (0-135)	25 (0-177)	16 (0-156)	7 (0-128)	8 (0-48)	15 (0-53)	15 (1-91)	15 (0-483)	10 (0-68)	8 (0-127)
	SS2	B	14 (1-324)	14 (0-55)	10 (0-40)	28 (2-520)	24 (0-368)	11 (1-404)	7 (0-53)	14 (0-94)	19 (2-263)	10 (0-152)	12 (1-221)	9 (0-81)
		A	15 (1-348)	15 (0-88)	9 (0-48)	28 (2-282)	22 (0-291)	11 (1-312)	7 (0-46)	13 (0-52)	21 (2-393)	10 (0-176)	13 (1-169)	9 (0-49)
6-8	SS1	B	24 (1-135)	12 (0-333)	12 (0-139)	31 (0-264)	23 (0-129)	9 (0-148)	11 (0-42)	12 (0-64)	19 (0-111)	15 (0-1264)	13 (0-68)	8 (0-117)
		A	22 (1-147)	13 (0-262)	13 (0-149)	32 (0-255)	24 (0-148)	9 (0-120)	10 (0-55)	12 (0-52)	20 (0-124)	16 (0-922)	13 (0-65)	8 (0-101)
	SS2	B	23 (1-344)	14 (0-56)	12 (0-27)	31 (0-318)	31 (0-256)	11 (0-461)	9 (1-35)	16 (0-60)	20 (1-329)	15 (1-171)	16 (1-123)	11 (0-59)
		A	25 (1-300)	15 (0-126)	12 (0-34)	30 (0-459)	32 (0-289)	10 (0-146)	9 (1-46)	14 (0-61)	22 (1-300)	14 (1-130)	17 (1-127)	12 (0-37)
9-12	SS1	B	22 (0-168)	14 (0-358)	12 (0-129)	39 (0-325)	27 (0-157)	9 (0-111)	10 (0-66)	17 (0-59)	23 (0-157)	21 (1-1032)	16 (0-56)	9 (0-99)
		A	22 (0-140)	13 (0-420)	12 (0-160)	40 (0-285)	27 (0-175)	9 (0-97)	10 (0-61)	18 (0-64)	25 (0-137)	21 (1-1230)	15 (0-82)	9 (0-109)
	SS2	B	29 (1-371)	16 (1-66)	11 (0-43)	37 (2-428)	36 (1-302)	13 (0-438)	7 (0-58)	16 (1-100)	29 (1-296)	17 (0-96)	18 (0-140)	11 (0-64)
		A	31 (1-277)	16 (1-101)	11 (0-74)	35 (2-258)	36 (1-234)	13 (0-274)	7 (0-54)	16 (1- 90)	26 (1-258)	17 (0-147)	20 (0-199)	10 (0-44)
12-16	SS1	B	22 (1-142)	12 (0-347)	9 (0-91)	41 (0-376)	26 (0-176)	9 (0-104)	9 (1-62)	15 (0-70)	25 (0-165)	21 (0-1067)	15 (0-70)	9 (1-110)
		A	22 (1-149)	13 (0-516)	9 (0-137)	36 (0-295)	26 (0-193)	9 (0-137)	9 (1-61)	15 (0-74)	22 (0-148)	23 (0-1235)	14 (0-86)	9 (1- 99)
	SS2	B	29 (1-272)	16 (1-78)	9 (0-42)	35 (2-343)	30 (0-258)	12 (1-529)	7 (0-60)	22 (1-96)	31 (0-269)	18 (1-109)	18 (0-112)	13 (0-74)
		A	26 (1-371)	16 (1-164)	9 (0-58)	40 (2-344)	29 (0-325)	12 (1-427)	8 (0-43)	20 (1-100)	31 (0-342)	16 (1-160)	19 (0-190)	12 (0-65)

Table 2: Values of the coefficient of variation (CV), mean, and 1% (P.1%) and 99% (P.99%) percentiles of the concentrations (μg·g^{-1}, †: ng·g^{-1}) for all elements in each of the sampling sites (SS1, SS2) in the washed (W) and unwashed (WW) samples of *Pseudoscleropodium purum* throughout the sampling period.

			Al	Cd†	Cr	Cu	Fe	Hg†	K	Na	Ni	Pb†	V†	Zn
SS1	W	CV (%)	31	22	51	20	30	16	18	45	32	89	21	14
		Mean	2300	290	21.0	8.50	580	73.0	4590	470	14.0	3635	9190	58.0
		P. 1 %	1010	158	8.90	6.10	305	58.0	3370	260	8.00	2550	5555	46.5
		P.99 %	4390	440	56.0	14.0	1110	125	7780	1240	30.0	9900	12625	78.0
	WW	CV (%)	45	28	99	15	54	14	17	42	63	92	24	15
		Mean	2220	285	21.0	8.30	630	72.0	4690	595	15.2	3505	9100	55.0
		P. 1 %	960	39.0	6.90	6.30	226	57.0	3370	335	7.00	2425	3840	44.0
		P.99 %	6770	470	166	11.5	2260	102	6500	1360	68.5	11100	13130	79.0
SS2	W	CV (%)	50	20	74	14	63	59	21	48	35	28	35	24
		Mean	330	54.5	7.60	6.10	202	37.5	6465	455	13.0	2100	2080	35.5
		P. 1 %	156	31.0	4.30	4.70	96.4	25.5	5150	240	5.05	880	1040	24.4
		P.99 %	900	81.0	39.0	8.10	770	143	10600	1110	25.5	3270	4445	56.0
	WW	CV (%)	48	21	59	17	53	70	19	43	30	26	30	21
		Mean	410	51.5	12.4	6.20	290	39.0	6600	485	16.2	2040	2475	32.5
		P. 1 %	178	33.0	6.20	4.05	136	30.0	5150	265	5.55	1070	1060	24.1
		P.99 %	1060	82.0	42.0	8.90	930	142	10400	1100	27.0	3405	4445	50.5

during the entire sampling period, although for most of the elements, the largest variations occurred at the end of the period, probably as a result of the effect of precipitation events on the concentrations of elements in the moss (Zechmeister et al., 2003, Couto et al., 2004). On one hand, rainwater may wash the moss, resulting in removal of the particulate material deposited on the surface of the plants, and resulting in a decrease in the amounts of contaminants deposited in this way. On the other hand, rainwater may dissolve elements adsorbed on the bryophyte tissues, thus facilitating their uptake by the organism. Accordingly, and taking into account that in the last 3 months of 2005, the mean precipitation in the region was higher than in the previous 30 years (Meteogalicia, Anuario Climatológico de 2005), it ap-

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pears likely that this was the cause of the fluctuations in the concentrations of the elements at the end of the sampling period.

Among the elements studied, the patterns of temporal variation in the concentrations of, for example, Cr, Fe and Ni, were very similar. This is probably because for this species of moss and in the study zone, these three elements are largely taken up from the soil (Fernández and Carballeira, 2001), where they are mainly deposited in particulate form. Furthermore, there appears to be a certain degree of seasonal influence in the concentrations of Na, consistent with the pattern observed by Couto et al. (2003), of a reduction in tissue concentrations of Na coinciding with the period of lowest productivity of the moss (in summer). The pattern was almost identical in SS1, located less than 1 km from the coast, and in SS2, located some 30 km from the coast. The concentrations of Hg increased by up to 5 times within 4 days in the uncontaminated site (SS2). This is consistent with the results obtained by Real et al. (2008), who observed short term episodes of contamination by Hg in Galicia, which affected the entire region. This is probably because Hg is a highly volatile metal (Bunt and Waanders, 2008) and may be transported long distances in the atmosphere; the whole region would therefore be affected by the emissions, which explains the peaks in this metal observed in the uncontaminated SS.

In summary, the percentage variation in the concentrations calculated for different periods, the CVs and the percentiles revealed very wide ranges of variation (Figs. 1 and 2). Likewise, as previously shown, the occasional peaks in concentrations were sometimes of considerable magnitude. This, combined with the weight of the error (in the total error) associated with the temporal variability indicates that the temporal variability in the concentrations of different elements in the moss may have a large effect on the results obtained in biomonitoring studies. We must take into account that although the errors associated with sampling and chemical analysis may be minimized (Fernández et al., 2002), the error associated with the temporal variability is inevitable and uncontrollable. The data obtained in sampling surveys carried out at specific times are therefore subject to a large degree

of uncertainty and the temporal representativeness of the results is very low.

One of the most important implications of the effect of the temporal variability in the concentrations of the elements in moss is related to the planning of extensive sampling surveys. Large numbers of samples should be collected throughout the year in order to provide temporally representative data. However, this is unviable in many cases as it would negate one of the great advantages of biomonitoring with terrestrial moss: the low cost of the method. Moreover, sampling over large areas requires several days or even weeks. This makes it impossible to obtain synchronized data, because if the variation in the concentrations occurs within a matter of days, comparison of samples collected several days apart would not be valid because the concentrations may have undergone large variations.

5. Conclusions

High frequency, long term sampling of the moss *P. purum* and subsequent analysis of the concentrations of 12 elements in the samples revealed that, on one hand, the concentrations of the elements varied greatly within short periods of time, and on the other hand, that there was a high degree of error associated with the temporal variability.

Many biomonitoring studies with terrestrial mosses are carried out in regional, national and even transnational networks, which are sampled annually or at best twice yearly. The results obtained in the present study demonstrated that the temporal representativeness of the results of such surveys is poor, given the high degree of uncertainty associated with the temporal variability. As improvement in the temporal representativeness of the data is almost impossible due to operational questions, we conclude that as the technique enables contaminated and uncontaminated sites to be differentiated, the results of biomonitoirng studies should be reinterpreted to provide qualitative or semiquantitative data on air quality, rather than providing absolute data with minimal temporal representativeness and with a high degree of associated uncertainty.

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CHAPTER II

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Growth rates and changes in heavy metal concentrations in segments of different ages in the terrestrial moss *Pseudoscleropodium purum*

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Abstract

We measured growth of the terrestrial moss *Pseudoscleropodium purum* during several well defined periods (monthly and quarterly between January 2008 and December 2009) at seven sampling sites affected by different climatic conditions. We also measured the concentrations of Cd, Cu, Hg, Pb and Zn in different moss segments comprising the tissue grown during each period. The concentrations of heavy metals in the different portions of moss shoots were highly variable, probably because of the high spatial and temporal variability in the growth rates of *P. purum* and the differences in the cation uptake/retention capacities of old and new tissues. The error associated with measurement of the concentrations of heavy metals in individual segments of the moss shoots ranged between 18-41%. The concentrations of Cd, Cu, Hg, Pb and Zn in the moss tissues appear to be more closely related to the physicochemical characteristics and the moss metabolism than to any preferential accumulation in young or old tissues. We conclude that comparison of the results of different biomonitoring studies is not valid, even when the same parts of the moss shoots are analyzed. Therefore, we recommend the use of green parts of moss shoots, as this would reduce the effect of tissue ageing on the cation uptake/retention capacity, although this prevents the establishment of a chronological relationship between the concentrations of heavy metals in moss tissues and atmospheric deposition.

Keywords: *Pseudoscleropodium purum, passive biomonitoring, air quality, moss growth, heavy metals.*

1. Introduction

Passive biomonitoring of air quality is based on the use of ubiquitous, abundant organisms that are able to take up and retain high levels of contaminants in their tissues. This reduces the costs of the chemical analysis and greatly improves the quality of the results in comparison with physicochemical analysis. Because of these advantages, passive biomonitoring, more specifically the moss technique, has been developed over the last 40 years to become a well established complement to physicochemical methods in the assessment of the atmospheric deposition of heavy metals worldwide (Chen et al., 2010; Wilkie and La Farge, 2011), especially in Europe (Harmens et al., 2010a). The moss technique is based on the use of organisms with a low capacity for metabolic regulation and with a high degree of structural simplicity (mosses generally lack a well developed vascular system, roots and cuticle, and their tissues usually comprise a single cell layer). However, mosses are living organisms and their suitability as biomonitors largely depends on their biology, which in turn depends on the surrounding environmental conditions (Zechmeister et al., 2003).

In some biomonitoring studies, processes related to the moss physiology limit the quality of the results. Thus, some authors have hypothesized the preferential accumulation of various elements in particular parts of moss tissues (generally leading to the accumulation of nutrients such as Na, Ca or K in apical parts of moss shoots and the accumulation of heavy metals such as Pb, V and Hg in basal parts) (Pakarinen and Rinne, 1979 in *Hylocomium splendens* and *Pleurozium schreberi*; Grodzinska et al., 1990 in *H. splendens* and *P. schreberi*; Bargagli et al., 1995 for *Pseudoscleropodium purum*; Leblond et al., 2004 in *P. purum*). Other authors have also demonstrated acropetal and basipetal movement of elements within moss shoots (Brown and Brown., 1990; Sidhu and Brown, 1996; Wells and Brown, 1996; Brümelis and Brown, 1997; Brümelis et al., 2004). Both types of processes would alter the linear relationship between the concentrations of heavy metals in the environment and in the portion of moss analyzed, as they both prevent

retention of the elements in the tissues in which they were first located after uptake.

The above findings have important implications for the passive biomonitoring of air quality, as the reliability of the technique is mainly based on the maintenance of the linear relationship between the exposure concentrations (atmospheric heavy metals deposition) and the tissue concentrations (concentrations obtained in the parts of the moss shoots taken for the analysis) (Reimann et al., 1999). However, the technique is still applied without a good knowledge of the dynamics of heavy metals within the moss tissues. In fact, no attempts have been made either to measure the concentrations of heavy metals in moss segments grown during well defined periods or to determine the effect of tissue ageing on the concentrations of heavy metals.

In the present study, we investigated the growth of *Pseudoscleropodium purum* at different sampling sites during several well defined periods. We also collected samples of the species and determined the concentrations of heavy metals, with the following objectives: i) to assess the annual growth rates of *P. purum* under different climatic conditions; ii) to study the within sampling site variability of the growth of *P. purum* throughout the study; iii) to assess the changes over time in the concentrations of various heavy metals in segments of different ages in moss shoots; and iv) to determine how moss growth and the temporal changes in the concentrations of heavy metals within moss tissues affect air quality biomonitoring studies with native terrestrial mosses.

2. Material and methods

Growth of the terrestrial moss *Pseudosceropodium purum* Hewd. M. Fleisch. was measured at seven sampling stations (SS) located in Galicia (NW Spain) (Fig. 1). Measurements were made quarterly at six of the SS (SS1-SS6) and monthly at the remaining SS (SS7), between January 2008 and December 2009. Samples of *P. purum* were collected at the same time as growth measurements were made.

2.1 Growth measurements

Moss growth was assessed in terms of length increments of the main stem, according to Russell (1984). Thus, 100 shoots of *P. purum* growing naturally at each SS were collected. Groups of ten shoots were tied to a thread, with each shoot tied at approximately 1 cm from the apex (see Fig. 1A). Each group of shoots was labelled and identified and each shoot within each group was also identified by assigning a number from 1 to 10 (from one end to the other). Finally, the shoots were placed within the moss carpet at each SS throughout the duration of the study.

The length of moss shoots was measured periodically (monthly or quarterly) by taking a photograph of each group of shoots against a background of graph paper (Fig. 1A). The shoot length was determined for each shoot and

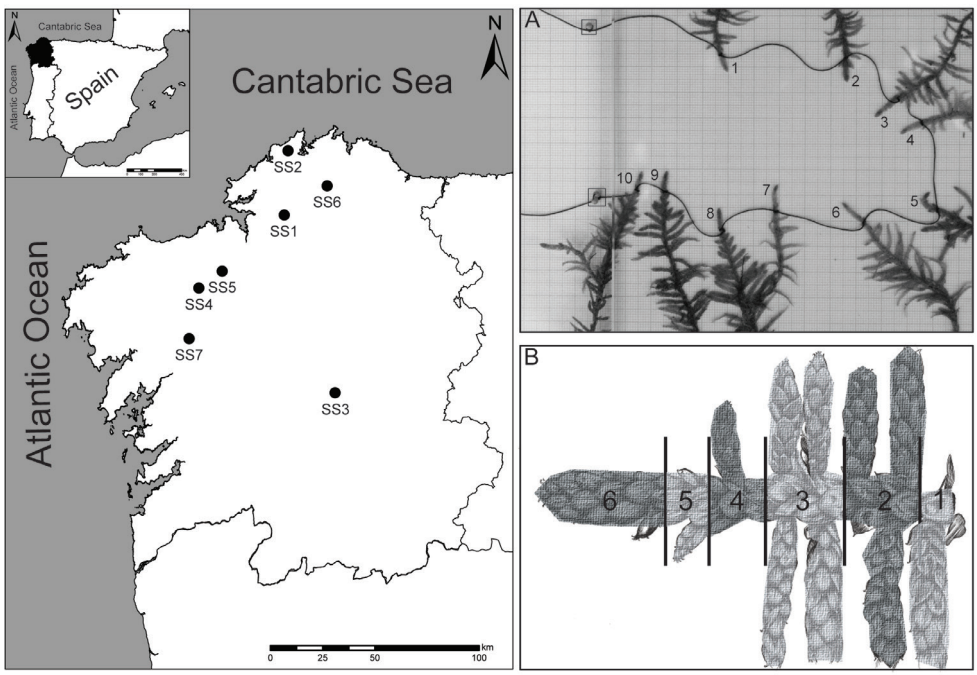


Figure 1: Map showing the location of the seven sampling sites (left hand side of the figure). The following are also shown (on the right hand side of the figure): A) photograph of the moss shoots placed on graph paper, showing the experimental design made for growth measurements; B) diagram showing the different segments cut from each moss shoot at sampling time 6. Each segment corresponds to the increase in length of the moss in the most recent (segment 6) and the previous three-month periods (segments 5 to 1).

time interval by using image processing software. For each SS, length increments were then calculated for each time period as the median difference in shoot length between one period and the preceding period. The error associated with measuring the length increments (percentage of error associated with the mean length increments for each time interval and SS), assuming a normal distribution, was determined. The error was less than 10% in most cases and was only exceptionally higher than 30%. The error in the length of the different segments cut (Fernández et al., 2010) was also determined (± 1 mm with respect to the desired length).

2.2 Sampling and processing

One sample of *P. purum* was collected from each SS and time interval (times 1 to 8 in the case of SS1-SS6 and times 1 to 23 in the case of SS7). When possible, samples were collected in open areas and comprised approximately 30 subsamples of similar weight and evenly distributed within a sampling area of approximately 30 x 30 m (Fernández et al., 2002, Aboal et al., 2006, Varela et al., 2010).

Once in the laboratory, whole moss shoots were separated from each other, and all the adhered material was removed. All shoots in each sample were cut into various segments, between the apex and the base, which corresponded to the length increments undergone by the moss at each time interval (Fig. 1). Thus, for samples SS1 to SS6 collected at time 8 (t8), each shoot was cut into 8 segments; the first segment (the apicalmost part of the shoot) corresponded to the length that moss had grown between September 09 and December 09 (the final measurement period in the study), and the other segments to the length that moss had grown in the previous three-month periods. For the sample collected in SS7 at t23, each shoot was cut into 22 segments (photographs from t7 were lost, and therefore the moss growth in this period was included in t8, resulting in a two month growth period), with the apicalmost segment corresponding to the length that moss had grown during November 09, and the other segments, corresponding to the length that moss had grown the previous months (for details, see Fig. 1).

The moss subsamples were then dried at 45ºC and homogenized in a tangential mill (metal free, Retsch MM 400).

2.3 Chemical Analysis

Heavy metals were extracted by acid treatment (2% HNO_3 v/v) and ultrasound (SONICS Vibra cell VCX 130). The concentrations of Cd, Cu, and Pb were determined by graphite furnace atomic absorption spectrometry (Perkin Elmer AAnalyst 600). The concentrations of Zn were determined by flame atomic absorption spectrometry (Perkin Elmer 2100), and the concentrations of Hg were determined in an elemental analyzer (Milestone DMA80).

For analytical quality control, duplicate samples were analyzed once every nine samples. Two certified reference materials: M2 and M3 (*Pleurozium schreberi*, Steinnes et al., 1997), were also analyzed once every nine samples. Analytical blanks were analyzed at the same frequency to control for possible contamination.

In general, the analytical quality of the process was satisfactory. The overall percentage error (Ceburnis and Steinnes, 2000) ranged between 3 and 7% for all elements. The percentage recovery ranged between 85% (for Hg) and 110% (for Pb) in M2, and between 77% (for Cu) and 114% (for Hg) in M3.

3. Results

The annual growth (March 2008-March 2009) of *P. purum* varied widely within the study region, ranging between 2.9–7.2 cm·year^{-1} at all the SS (Table 1). Moss growth also varied widely within the same SS throughout the study, as shown by the large differences in the length increments of the main stem between periods of time of the same duration (Table 1). Thus, for example, in the case of SS5, the maximum increment for a three month period was 4.6 cm, whereas the minimum increment was 1.2 cm. In the case of SS7, the monthly growth of *P. purum* (detailed data not shown) was also highly variable, with maximum growth of 0.8 cm and a minimum of 0.13 cm. However, maximum and minimum growth did not occur at specific times (maximum

values occurred during different three-month periods: April-May-June, July-August-September and October-November-December; minimum values also occurred in different periods: January-February-March, July-August-September, and October-November-December).

Table 1: Main stem length increments (cm) in *Pseudoscleropodium purum* for the three-month period at the seven sampling stations (dates correspond to each collection). Annual growth at each SS (between March 2008 and March 2009) is also shown. Underlined values: maximum and minimum length increments for a three-month period at each SS.

	Jun-08	Sep-08	Dec-08	Mar-09	Jun-09	Sep-09	Dec-09	Annual growth
SS1	2.2	2.2	0.7	0.7	0.9	0.4	0.8	5.8
SS2	1.6	1.3	0.8	0.9	2.7	1.5	0.4	4.6
SS3	2.7	0.9	2.6	0.9	0.7	0.2	0.8	7.1
SS4	1.5	1.7	1.0	0.5	1.1	0.3	-	4.8
SS5	2.3	1.6	2.1	1.2	4.6	3.3	-	7.2
SS6	1.8	0.5	0.4	0.2	1.1	1.0	1.7	2.9
SS7	1.4	0.6	1.9	0.8	1.2	0.7	1.4	5.2

The times required for *P. purum* to grow approximately 3 cm in length (the measure commonly used in biomonitoring studies) at each SS are shown in Table 2. At SS1, SS3 and SS4, the moss shoots grew approximately 3 cm in length in 12 months. However, at SS5, SS6, SS7 and SS7 sampled monthly, the moss shoots grew this length within 3, 6, 9 and 8 months respectively (data for SS2 were not taken into account because at this SS *P. purum* only grew 1.86 cm in 6 months, but after 9 months it had grown 4.5 cm: both values are too far from 3 cm). Therefore, the period that the apicalmost three cm of moss shoots had been exposed to atmospheric heavy metal deposition ranged between 3 and 12 months, depending on the SS.

Taking into account that the heavy metal concentrations were determined separately in the different segments cut from the moss shoots at each sampling time (from apex to base, corresponding to the length increment undergone by the moss in the previous three/one-month period), the con-

centrations of heavy metals in moss shoots from samples collected in December 2009 were obtained in the following: i) the apicalmost 3 cm of moss

Table 2: The time (months) required for *Pseudoscleropodium purum* to grow approximately 3 cm in length (the exact length increments, in cm, are shown between brackets) is shown for each SS. Mean concentrations of heavy metals weighted by length ($ng \cdot g^{-1}$; †: $\mu g \cdot g^{-1}$) in the three apical cm of the main stem, in the portion of moss grown during 12 months, and in the whole shoot are also shown for each SS. Tissue quantity: length, in cm, of each portion considered (3 apical cm, 12 months growth or whole shoot); SS7: data obtained with the measurements made monthly in SS7.

SS	Time vs growth	Shoot portion	Cd	Cu†	Hg	Pb	Zn†	Tissue quantity
SS1	12 (2.81)	3 cm	19.6	3.3	13.6	833	25	2.8
		12 months	19.6	3.3	13.6	833	25	2.8
		whol e shoot	62.1	8.7	55.1	4559	62.5	8.9
SS2	6 (1.89)	3 cm	28.4	3.2	12.2	255	19.9	1.9
		12 months	75.9	6.3	36.3	1425	52.5	5.5
		whole shoot	128.9	12.1	83.3	4547	90.9	10.1
SS3	12 (2.64)	3 cm	43.7	2.4	11.8	261	21.2	2.6
		12 months	43.7	2.4	11.8	261	21.2	2.6
		whole shoot	174.2	7.7	49.7	2470	70.1	9.9
SS4	12 (2.94)	3 cm	42.2	3.7	13.5	719	19.5	2.9
		12 months	42.2	3.7	13.5	719	19.5	2.9
		whole shoot	100.4	8.5	40.3	3232	45.9	7.3
SS5	3 (3.35)	3 cm	11.2	1.3	12.5	342	11.4	3.3
		12 months	46.5	5.1	45.9	1695	37.5	11.3
		whole shoot	52.6	5.7	54.2	2088	42.9	12.9
SS6	6 (2.65)	3 cm	32.3	2.4	12.1	315	13.1	2.6
		12 months	45.3	3.8	17.6	655	18.2	3.9
		whole shoot	83.9	7.9	38.1	2130	33.7	7.6
SS7	9 (2.7)	3 cm	34.9	6.9	17.3	2264	20.9	2.7
		12 months	59.8	11.4	33.4	4738	32.6	4.6
		whole shoot	117.8	17.1	73.6	7655	57.1	8.4
SS7	8 (3.28)	3 cm				1769		3.3
		12 months				3459		4.8
		whole shoot				8286		9.9

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shoots; ii) the portion of moss grown during the previous 12 months; and, iii) the whole shoot. As expected, the concentrations of all the elements in the whole shoot were considerably higher than those in the other portions (Table 2). In the case of SS5, SS6, SS7 and SS7 sampled monthly, concentrations in the 12 month portions were between 1.5 and 5 times higher than in the apicalmost 3 cm of moss shoots (Cd, Hg and Zn in SS6 and Pb in SS5 respectively).

Figure 2 shows the concentrations of Cd, Cu, Hg, Pb and Zn in segments of moss shoots of different ages (starting at 3, 6 and approximately 9 months old in September 2008), at each SS and sampling period. In the case of SS7, which was sampled monthly, quarterly data on the concentrations of heavy metals were obtained by combining the subsamples of the segments from the three months corresponding to each period to make a composite sample. There appeared to be two different patterns concerning the heavy metal concentrations and age of the segments. Firstly, there was no apparent trend in the concentrations of Cd, Cu and Zn in young and old segments (sometimes they were higher in old tissues than new tissues and vice versa i.e., the concentrations were not age dependent). However, the concentrations of Hg and Pb tended to be higher in old tissues, although the levels in old and new tissues were sometimes very similar (or even higher in new tissues). Secondly, the concentrations of all heavy metals varied considerably within the same segment throughout the study (Fig. 2). The variations were similar in all segments, independently of their age. Thus, when concentrations in the older segments increased/decreased, the same was also observed in the intermediate-aged and younger segments (i.e. Cd concentrations in SS2 increased almost 2 times in the three segments during the period June-Sept 09; Zn concentrations in SS6 decreased 2, 3 and more than 3 times in the young, middle and old segments respectively during the period Sept-Dec 09).

The concentrations of Pb were also determined in the segments of different ages (6, 5, 4, 3, 2 and 1 months age respectively in June 2008) corresponding to the growth measurements and sampling collections made monthly

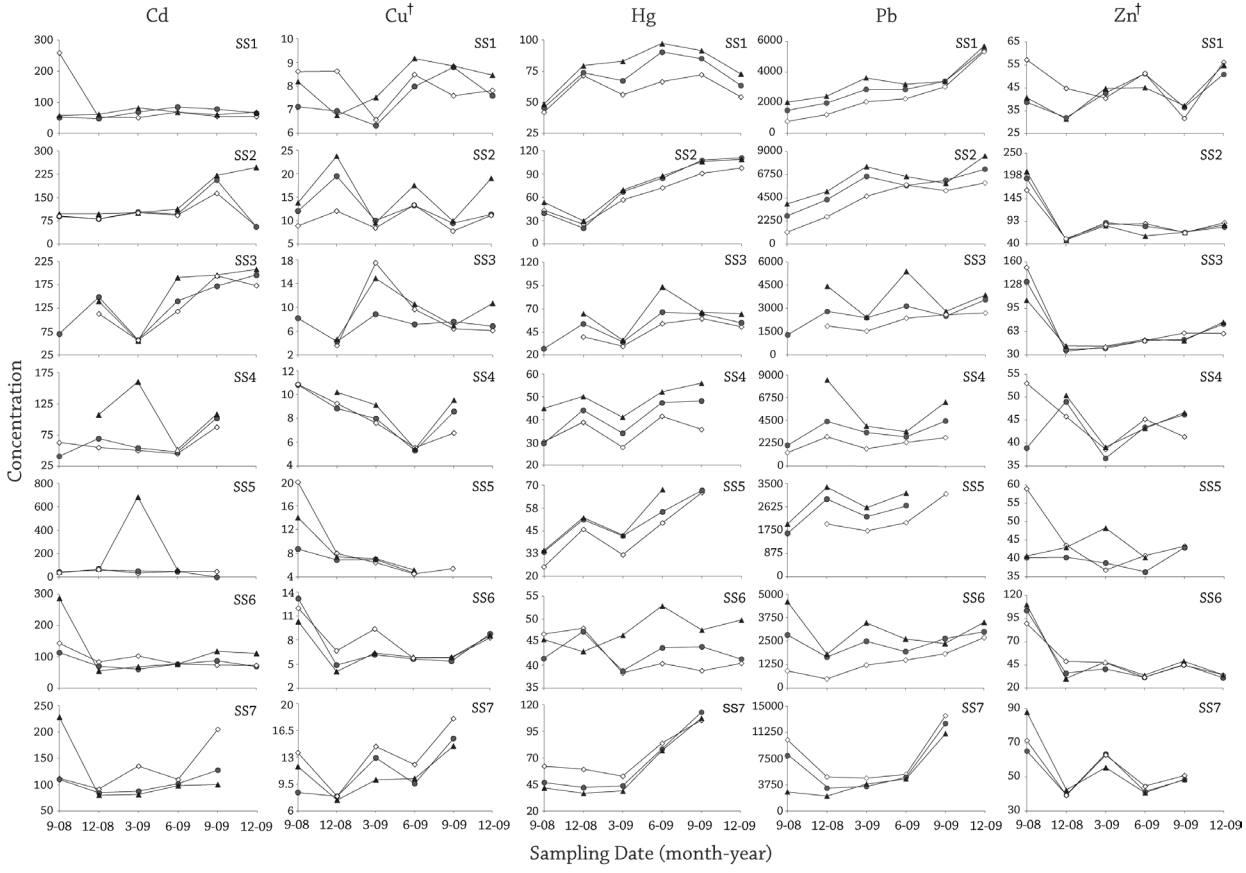


Figure 2: Changes in the concentrations ($ng \cdot g^{-1}$; †: $\mu g \cdot g^{-1}$) of heavy metals over time in segments of different ages in *Pseudoscleropodium purum*. Open rhombi: heavy metal concentrations in the youngest segment (3 months old in Sept-08); grey circles: heavy metal concentrations in the intermediate-aged segment (6 months old in Sept-08); closed triangles: heavy metal concentrations in the oldest segment (9 months old in Sept-08).

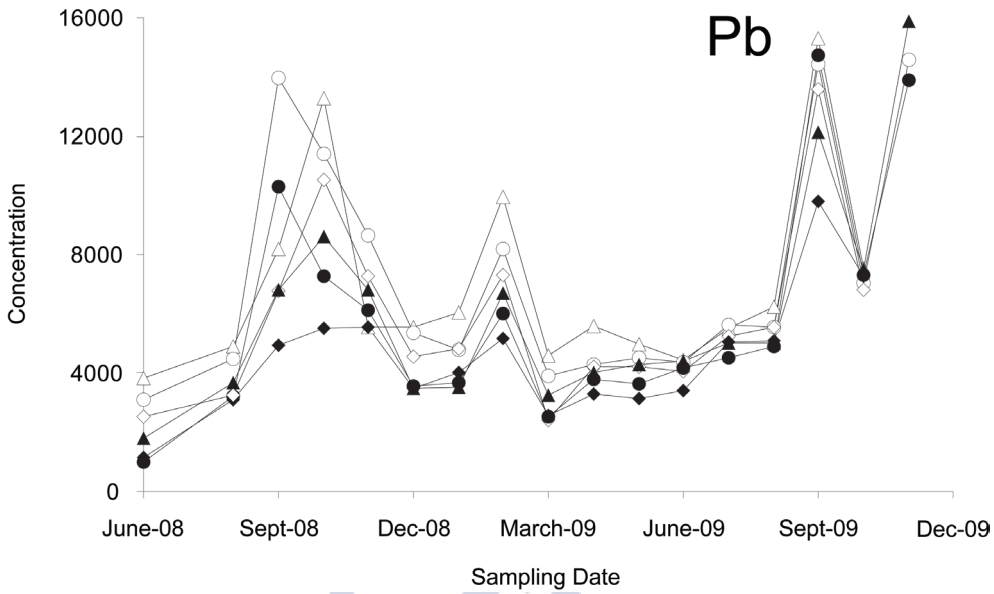


Figure 3: Changes in Pb concentrations ($ng \cdot g^{-1}$) over time in segments of different ages in *Pseudoscleropodium purum* collected monthly at SS7. Open triangles: Pb concentrations in the 6-month-old segment in June-08; open circles: Pb concentrations in the 5-month-old segment in June-08; open rhombus: Pb concentrations in the 4-month-old segment in June-08; filled triangles: Pb concentrations in the 3-month-old segment in June-08; filled circles: Pb concentrations in the 2-month-old segment in June-08; filled rhombus: Pb concentrations in the 1-month-old segment in June-08.

in SS7 (Fig. 3). This figure shows that the pattern of variation of Pb within moss tissues is also maintained even when data were obtained monthly instead of quarterly. As with the data from the three-month periods, the Pb concentrations tended to be higher in the older than in the younger tissues (although not always).

Finally, the mean difference between the concentrations of each heavy metal and sampling date in the youngest segment (open rhombi in Fig. 2) and in the intermediate-aged segment (grey circles in Fig. 2) was divided by the total variability in the concentration of each heavy metal in the youngest segment (maximum concentration minus minimum concentration registered throughout the experiment). This provided a measure of the mean error in the determination of the concentrations of each heavy metal in the youngest segment instead of in the intermediate-aged segment (approximately

30, 25, 26, 41 and 18% for Cd, Cu, Hg, Pb and Zn respectively).

4. Discussion

Assessment of the growth of *P. purum* in this study showed that the rate of annual growth of this species in the study region was between 1.3 and 3.3 times higher than previously reported for the same species (approximately 2.2 cm·year^{-1} in countries such as France and the United Kingdom; Kilbertus, 1968; Bates, 1987; Leblond et al., 2004). These results also reveal a very high within-SS variability in the length increments of the main stem for this species between periods of the same duration. Both results can be explained by the effect of the climatic conditions on moss physiology. Some authors have found positive correlations between moss growth and the mean annual temperatures and humidity in the particular study region (Pakarinen and Rinne, 1979; Zechmeister, 1995, 1998). Therefore, the generally more favorable climatic conditions in the present study region (NW Spain) than in northern regions of Europe (France and United Kingdom) may explain the higher annual growth rate of *P. purum* found in this study. However, it is also possible that growth rates will be more variable when climatic conditions are more variable (Zechmeister, 1998). The high degree of spatial and temporal variation in the moss growth observed here may be due to such heterogeneity.

The lack of seasonality in the maximum and minimum periods of growth of *P. purum* in this region may be due to the fact that moss physiology depends to a greater extent on small scale specific climatic variables (i.e. microsite temperature, humidity, solar radiation, etc.) than on the overall climatic conditions (Zechmeister, 1995). This would prevent the exact repetition of the same growth pattern from one year to the following in the same site and would explain the observed differences in the time required for the moss shoots to grow approximately 3 cm length in the study areas (3 to 12 months, see Table 2). Thus, moss growth cannot be extrapolated/predicted from year to year, site to site or from one species to another, but must be specifically measured.

The intra-specific variation in the growth rates of *P. purum* appear to constitute an important source of variability in the concentration of heavy metals found in its tissues, as previously observed by Malmer (1988) in *Sphagnum* mosses. This variability is partly generated because the moss growth rates determine the time that a portion of tissue of this species has been exposed to the atmospheric deposition of heavy metals. In this study, the period that the upper 3 cm of moss shoots had been exposed to atmospheric heavy metal deposition ranged between 3 and 12 months, depending on the SS (Table 2). Furthermore, the moss growth determines the relationship between old tissues/new tissues in the portion of moss used for the analysis in biomonitoring studies. This relationship will determine the heavy metal uptake capacity in mosses, as young and old tissues differ in their degree of maturation and, therefore, in the following characteristics: i) cell wall/cytoplasm ratio, which is higher in new young tissues and leads into a higher specific surface of absorption of heavy metals than in old tissues (Brown and Brown, 1990); ii) active uptake processes, which are faster in new and metabolically more active tissues (Fernández et al., 2010); iii) cation exchange capacity, which is higher in old and more mature tissues. This is due to the decrease in the degree of methylation of galacturonic acids from the cell wall during the maturation process, which leads to an increase in the number of cation exchange places available and therefore, a higher cation exchange capacity (Brown and Brown, 1990). Different proportions of old/new tissue would result in highly variable concentrations of heavy metals, which would explain the large differences observed in the concentrations of Cd, Cu, Hg, Pb and Zn in the various parts of moss shoots analyzed in this study (Table 2). Taking into account all of the above, we can assume that accurate estimation of the time that a portion of moss shoots has been exposed to the atmospheric deposition of heavy metals is not possible and that the concentrations of heavy metals obtained in biomonitoring studies largely depend on the portion of moss selected for the analysis. However, although this has also been found for other moss species (growth of which is not defined by annual segments) frequently used in biomonitoring studies (genus *Sphag-*

num in Malmer, 1988; *Pleurozium schreberi*, *Hypnum cupressiforme* and *Abietinella abietina* in Zechmeister, 1995, 1998), statements such as the following are still made: *"Shoots of moss corresponding to the last 3 years growth, except the current year, were sorted for analysis. The results from the 1996 campaign thus represent the average deposition for the years 1993-1995…"* (Galsomiès et al., 1999 using *Pleurozium schreberi*, *Hypnum cupressiforme*, *Pseudoscleropodium purum* and *Thuidium tamariscinum*); *"Only the last three-year's worth of growths of the moss materials was used…"* (Bačeva et al., 2012 for *Hypnum cupressiforme*, *Campothecium lutescens* and *Homalothecium sericium*); *"Only the last three years' growth of moss material was used for the analyses"* (Harmens et al., 2012 for *Pleurozium schreberi*, *Hypnum cupressiforme* and *Pseudoscleropodium purum*); *"…the green shoot sections representing the last 2 years growth were separated from the brown ones…"* (Manninen et al., 2013 for *Pleurozium schreberi*)

The concentrations of Cd, Cu, and Zn in segments of moss shoots of different ages at each SS and sampling period (Figs. 2 and 3) show that there is no preferential accumulation of these elements in young or old tissues of *P. purum*. Several authors have stated that nutrients such as Ca, Na, K and Mg accumulate preferentially in young tissues, while heavy metals tend to accumulate in old tissues (Pakarinen and Rinne, 1979; Bates and Farmer, 1990; Brown and Brown, 1990; Grodzinska et al., 1990; Bargagli et al., 1995; Wells and Brown, 1996; Brümelis and Brown, 1997; Leblond et al., 2004). However, according to the results of the present study, it seems that the higher/lower concentrations of heavy metals in old/young tissues is more closely related to the physicochemical characteristics of the elements and the characteristics of moss tissues, than to preferential accumulation. Thus, according to Nieboer and Richardson (1980), elements such as Cd and Zn are highly mobile within moss tissues, which would explain why their concentrations are independent of the age of the segment. In the case of Zn, which is a micronutrient, the concentrations may also depend on the metabolic status of moss tissues (Brown and Brümelis, 1996). Other elements such as Hg and Pb form strong bonds that greatly reduce their mobility within moss tissues.

This explains the general tendency of these elements to occur at higher concentrations in older segments and also why they sometimes occur at higher levels in young tissues (their low mobility would prevent their mobilization towards other parts of moss shoots; see Figs 2 and 3). The concentrations of Cu followed a similar pattern as those of Cd and Zn, even though the mobility of Cu within moss tissues is low (Brown and Brümelis, 1996), possibly because of its role as a micronutrient.

The concentrations of all the heavy metals in the different segments of *P. purum* varied considerably over time, independently of their age. Moreover, there was no positive relationship between the concentrations of the heavy metals studied and the time of exposure, and there was also no temporal pattern in the increases/decreases in the concentrations (Figs. 2 and 3). Thus the whole shoot is influenced by the factors that affect the equilibrium established between the moss and the environment in the same way. This is not surprising if we assume that the moss does not integrate all the elements that it receives, but that the inputs and outputs of elements depend on different factors related to the moss biology and the environment (Couto et al., 2004; Boquete et al., 2011).

Finally, the most important implication of the data presented and discussed here is that the results of biomonitoring studies carried out using the same portion of moss shoots from the same species, but collected in different regions (even if the samples are collected on a local scale), are not comparable. This is because such studies involve samples with different growth patterns and that have grown under different equilibrium conditions. This can lead to large differences in the concentrations of heavy metals that reflect differences generated by growth and other processes rather than differences in the inputs of the elements that the mosses have received in the environment in which are growing.

Measurement of moss growth at each SS (to prevent this factor affecting the results) is not viable for all biomonitoring studies. One possible solution could be to collect species such as *H. splendens* that generate well defined annual growth segments, as recommended by the ICP Vegetation monitoring

protocol (Monitoring Manual/2010 Survey; Harmens et al., 2010b). However, Brown and Brümelis (1996) pointed out that age-related differences in heavy metal concentrations also occur in *H. splendens* due to differences in the quality and quantity of the cell wall material and in the metabolic behavior and cation requirements of the cell during maturation. These authors stated that the mean concentration of a multi-aged segment (i.e. the segments corresponding to the last 3 years of growth - as recommended in the ICP Vegetation protocol for *H. splendens*) would be an oversimplification of the real temporal pattern of heavy metal accumulation in moss tissues.

According to all the information obtained in this study, and assuming that the time that mosses have been exposed to the atmospheric deposition of heavy metals cannot be indicated, we conclude that the use of the green parts of moss shoots (i.e. including live and more active tissues) is the best option in passive biomonitoring studies. Firstly, because the error associated with determining the concentrations of heavy metals in one segment and not in other (in this case, in the youngest segment and not in the intermediate-aged segment) ranges between 18% and 41% for all the elements studied here, and secondly, because use of this part of the tissue reduces the effect of tissue ageing on the cation uptake/retention capacity.

5. Conclusions

The high degree of spatial and temporal variability of *P. purum* growth precludes exactly the same growth patterns occurring from year to year, even within the same SS. This prevents accurate estimation of the period of time that the moss has been exposed to the atmospheric deposition of heavy metals, and it leads to highly variable concentrations of these elements in the different parts of moss shoots analyzed in biomonitoring studies.

Furthermore, the concentrations of Cd, Cu, and Zn are not age dependent in *P. purum*, so that this species does not preferentially accumulate these elements in old/young tissues. The tendency of Hg and Pb to be more abundant in old tissues also appears to be more closely related to the lower mobility than to the preferential accumulation of these elements.

Moreover, the lack of a positive relationship between Cd, Cu, Hg, Pb and Zn concentrations in moss tissues and the time of exposure clearly indicates that *P. purum* does not integrate these heavy metals. Thus, the whole shoot (both young and old tissues) appears to be influenced by the equilibrium established between the moss and the environment, and the concentrations of elements will increase/decrease according to the factors that affect this equilibrium (chemical, biological and environmental factors).

In light of the above, we conclude that comparison of the results of biomonitoring studies that even use the same portion of moss shoots is not valid (even when comparing species with well defined annual growth segments). Therefore, we recommend the use of green parts (i.e. live tissues) of moss shoots. This would reduce the effect of tissue ageing on the cation uptake/retention capacity, although it would prevent the establishment of a temporal relationship between the concentrations in moss tissues and the atmospheric deposition of heavy metals.

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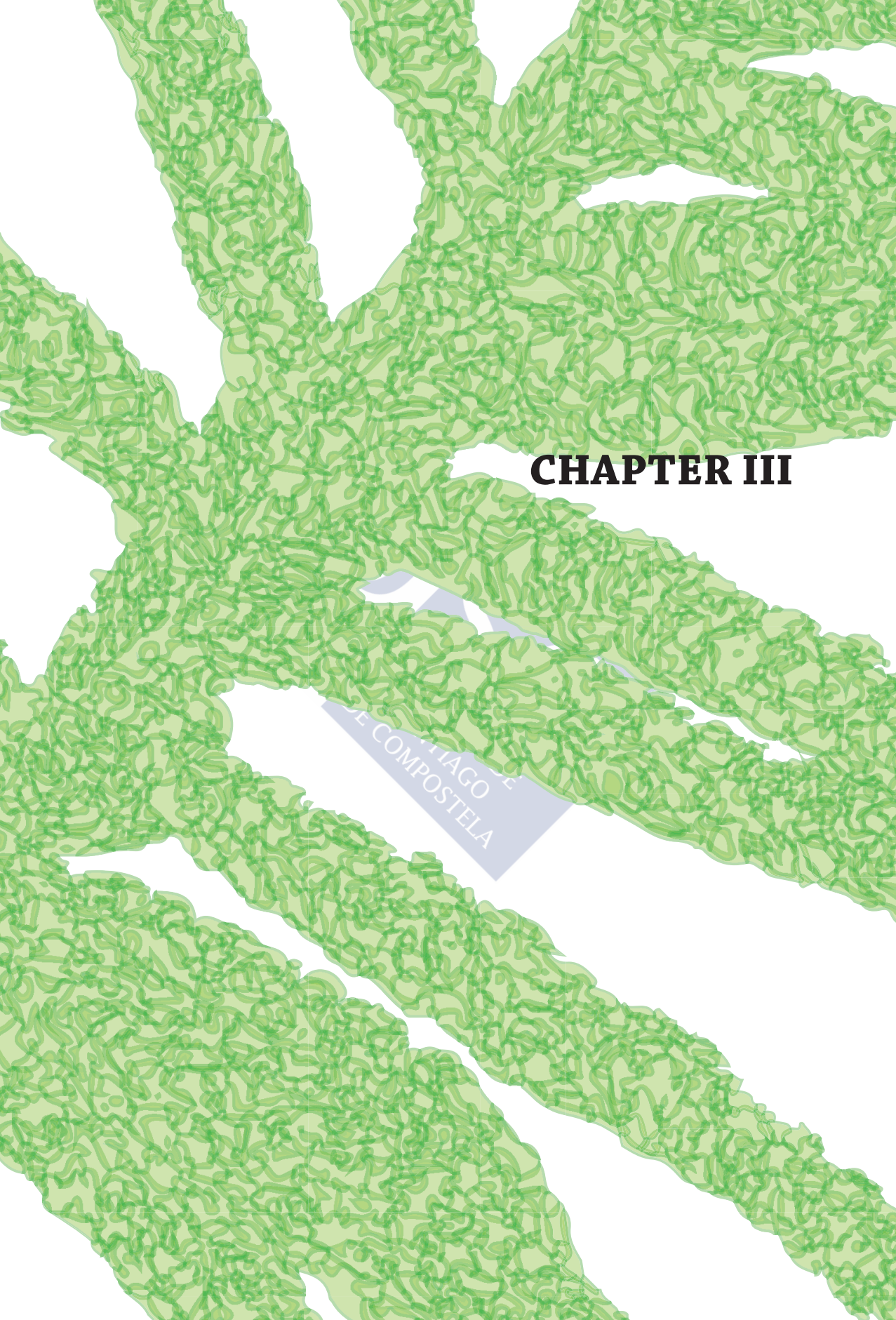
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CHAPTER III

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Assessing the tolerance of the terrestrial moss *Pseudoscleropodium purum* to high levels of atmospheric heavy metals: a reciprocal transplant study

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Abstract

We measured the concentrations of Cd, Cu, Hg, Pb and Zn in samples of the terrestrial moss *Pseudoscleropodium purum* reciprocally transplanted between an unpolluted and two polluted sampling sites. At the beginning of the experiment, the concentrations of all these elements differed significantly between mosses from the unpolluted site and moss from the polluted sites. In general, the concentrations of the heavy metals in mosses from both polluted sites transplanted to the unpolluted site decreased until they reached the same levels as in autotransplants at this site (after 480 - 840 days). However, the concentrations of all heavy metals in mosses transplanted from the unpolluted site to both polluted sites increased to higher levels than in the autotransplants (except for Cu, Hg and Pb at one of the sampling sites). These results lead us to conclude that mosses that have been continuously exposed to high atmospheric deposition of heavy metal undergo an adaptive response (probably genotypic) to such conditions. We therefore recommend active rather than passive biomonitoring of air quality in industrial environments and also recommend further investigation into the mechanisms involved in this response.

Keywords: *Pseudoscleropodium purum, adaptation, biomonitoring, air quality, atmospheric deposition.*

1. Introduction

A biomonitor is an organism (or any biological entity) that provides quantitative information about qualitative aspects of the environment, i.e. it produces measurable responses to changes that occur in the environment in

which it grows (Markert et al., 1999). Different types of biomonitors have been described; e.g., some biomonitors accumulate contaminants from the surrounding environment and the concentrations can then be measured in their tissues. Ideal organisms for use as this type of biomonitor should maintain a linear relationship between the concentrations to which they are exposed and the concentrations accumulated in their tissues. Only such a linear relationship will allow organisms to provide unequivocal information about their environment (Markert et al., 1999). However, many organisms do not fulfil this requisite, either because they act as "excluders" (maintaining low bioconcentration factors in the presence of high levels of contaminants in the environment) or because they act as "hyperaccumulators" (maintaining higher bioconcentration factors in response to low environmental concentrations) (Baker, 1981).

Terrestrial mosses, which are examples of accumulator organisms used as biomonitors, are often used to estimate atmospheric deposition of heavy metals (Berg and Steinnes, 1997; Berg et al., 1995; Fowler et al., 2006; Ross, 1990; Schintu et al., 2005; Thöni et al., 1996). However, the possible existence of mechanisms of heavy metal exclusion in moss exposed to high concentrations of these elements was not considered in any of these studies. Such mechanisms, which would increase the tolerance of moss to contamination, would alter the linearity of the relationship between exposure concentration and body burden and would therefore place in question the suitability of mosses as biomonitors.

Some of the mechanisms of heavy metal exclusion have been described in higher plants. Thus, Cumming and Taylor (1990) found that decreased binding of various metals to cationic exchange sites was due to genetic variation in the structure of transport proteins in the cell membrane. According to this, metal accumulation may be blocked by changes in the molecular properties of proteins located in the membrane (Fitter and Hay, 1987). Another possible mechanism of metal exclusion, described in higher plants, is the active transport of metal cations out of the cytoplasm (Larcher, 1995). However, studies concerning mechanisms of heavy metal exclusion in mosses

are very scarce and only refer to species that are not usually used as biomonitors; e.g., Shimwell and Laurie (1972) observed that *Dicranella varia* can excrete most of the Pb and Zn from the gametophyte in a powdery crust on the moss carpet. Nonetheless, differences in heavy metal tolerance between different populations of the same species have been reported (Jules and Shaw, 1994; Shaw et al., 1987; Shaw, 1988; Wells and Brown, 1995). According to several authors, there are two possible mechanisms explaining the above-mentioned differences: i) genetic adaptation, i.e., genetic differentiation between populations of the same species in response to selective pressure (i.e. exposure to high concentrations of heavy metals), thus giving rise to different ecotypes of the same species (Jules and Shaw, 1994; Shaw et al., 1987); and ii) physiological acclimation, whereby the species display sufficient phenotypic plasticity to enable them to adapt to environments with unfavourable conditions without the need for genetic differences (Shaw, 1990).

It was previously thought that bryophytes displayed limited genetic variability because of the dominance of the haploid gametophytic stage in their life cycle and because they often reproduce asexually. Some authors stated that bryophytes were organisms with a slow rate of evolution and genetically depauperated (Anderson, 1963; Crum 1972, 1973). However, this view changed when Cummings and Wyatt (1981), who carried out isoenzyme studies, found an unexpectedly high amount of genetic variation in bryophytes, comparable to that found in higher plants. As genetic variability may be considered as a measure of evolutionary potential (Shaw et al., 1989) and of the capacity of live organisms to adapt to unfavourable environments, mosses may possess a much greater capacity to adapt (whether phenotypically or genotypically) to environments with high concentrations of heavy metals.

In studies involving the simultaneous use of native and transplanted mosses to biomonitor atmospheric deposition of contaminants, it has been hypothesized that the observed differences may be due to tolerance mechanisms. Thus, in a study of *Pseudoscleropodium purum* carried out in the surround-

ings of an electricity power plant, and in order to explain differences in uptake of elements, Fernández and Carballeira (2000) hypothesized that the native moss may adapt to the environments in which it grows by blocking the uptake of contaminants. Likewise, in a study with the same moss, carried out in the surroundings of a chlor-alkali plant, Fernández et al. (2000) suggested that the native moss adapts to the emissions, because at the same distance from the focal point of contamination, the levels of Hg were much higher in the transplanted moss than in the native moss. More recently, Samecka Cymerman and Kempers (2007) compared the bioconcentration of some heavy metals in *Pohlia nutans* transplanted from a control to a polluted site with bioconcentration in species growing naturally in both sites. They found that concentrations of heavy metals in transplanted mosses were significantly higher than those of native mosses from the polluted site, and they concluded that transplanted mosses better reflected the level of contamination. Finally, in a study in which specimens of *Hylocomium splendens* were transplanted between polluted and control sites, Tabors et al. (2004) concluded that there appear to be inherent differences in the capacity of mosses from uncontaminated and contaminated sites to adsorb and accumulate heavy metals.

The presence of mechanisms of heavy metal tolerance in moss, similar to those described for higher plants, would be an important limitation to the use of mosses for biomonitoring the atmospheric deposition of these elements because the requisite of a simple linear correlation between the concentrations of the contaminant in the environment and in the organism would not be fulfilled (Bargagli, 1998; Markert et al., 1999). Moreover, it would not be possible to compare the results obtained in biomonitoring studies carried out at regional, national or transnational scales, because these involve environments affected by different degrees of contamination, in which the native mosses may possess different capacities to accumulate heavy metals.

Although it has been suggested that mosses may adapt to heavy metal contamination in industrialized areas, no specific studies have been carried out

to confirm this hypothesis. Therefore, in the present study, we transplanted specimens of moss from polluted areas to an unpolluted area, and vice versa, to test for any differences in the accumulation of heavy metals in mosses between the different areas. We discuss the implications of the possible adaptations exhibited by mosses growing in zones with high loadings of atmospheric contaminants on the use of native mosses in biomonitoring studies.

2. Material and Methods

2.1 Sampling

Samples of the terrestrial moss *Pseudoscleropodium purum* (Hedw.) M. Fleisch were reciprocally transplanted between three sampling sites (SS) in Galicia (NW Spain): an unpolluted (UP) site and two sites located in the surroundings of focal points of contamination (P1 was close to an iron smelter and P2 was close to an aluminium smelter) (Fig. 1). Moss samples were transplanted from UP to the other two SS and vice versa. Samples of the moss growing naturally at each SS were also "autotransplanted" (i.e. transplanted within the same sites).

Moss transplants and autotransplants were prepared by isolating small sections of moss mat in frames of dimensions 15 x 10 cm x 3 cm (Cenci, 1998). Shallow holes of the same dimensions as the frames were made in the soil and the prepared moss transplants were placed inside the holes. Three replicates of each moss transplant and autotransplant were made at each SS and for each exposure period. Samples were collected 30, 60, 120, 240, 480 and 840 days after the start of the experiment, in February 2008 (for a detailed scheme of the sampling, see Fig. 1). Some samples were lost during the experiment (all replicate samples transplanted from P1 to UP for the 840 day exposure period and two of the replicate samples transplanted from UP to P1 for the same exposure period).

2.2 Processing and Chemical Analysis

Prior to analysis, the samples were cleaned and apical segments (3-4 cm

length) were cut from the shoots; any material remaining adhered to the segments was removed manually. The samples were maintained in a moisture-saturated atmosphere at 10ºC for one week, to ensure membrane integrity. The samples were then washed with bidistilled water, with shaking, for 30 sec and dried in an oven at 45 ºC. Finally, the samples were homogenized in an ultracentrifuge mill (Retsch ZM100), and the metals were extracted by acid treatment (2% HNO_3 v/v) and ultrasound (SONICS Vibra cell VCX 130). The concentrations of Cd, Cu, Pb and Zn were determined by graphite furnace atomic absorption spectrometry (Perkin Elmer AAnalyst 600) and, when necessary, by flame atomic absorption spectrometry (Perkin Elmer 2100). The concentrations of Hg were determined in an elemental analyzer (Milestone DMA80).

For analytical quality control, duplicate samples were analyzed once every nine samples for measurement of Cd, Cu, Pb and Zn. For Hg, duplicate samples were analyzed each time. Two certified reference materials: M2 and M3 (*Pleurozium schreberi*, Steinnes et al., 1997), were also analyzed once every nine samples. Analytical blanks were also analyzed, at the same frequency, to control for possible contamination.

In general, the analytical quality of the process was satisfactory. The overall percentage error (Ceburnis and Steinnes, 2000) ranged between 2 and 10% for all elements, except Pb (12%). The percentage recovery in M2 ranged between 82% (for Hg) and 111% (for Pb) for all elements, except Zn (73%). In the case of M3, the percentage recovery ranged between 81% (for Zn) and 114% (for Hg) in all cases.

2.3 Statistical Analysis

Differences in heavy metal concentrations between moss transplants and autotransplants for each SS and sampling time were assessed by a two sample t-test for independent samples ($P<0.05$) with the provenance as the grouping variable and heavy metal concentration as the dependent variables. All statistical analyses were performed with IBM SPSS statistics 20 software.

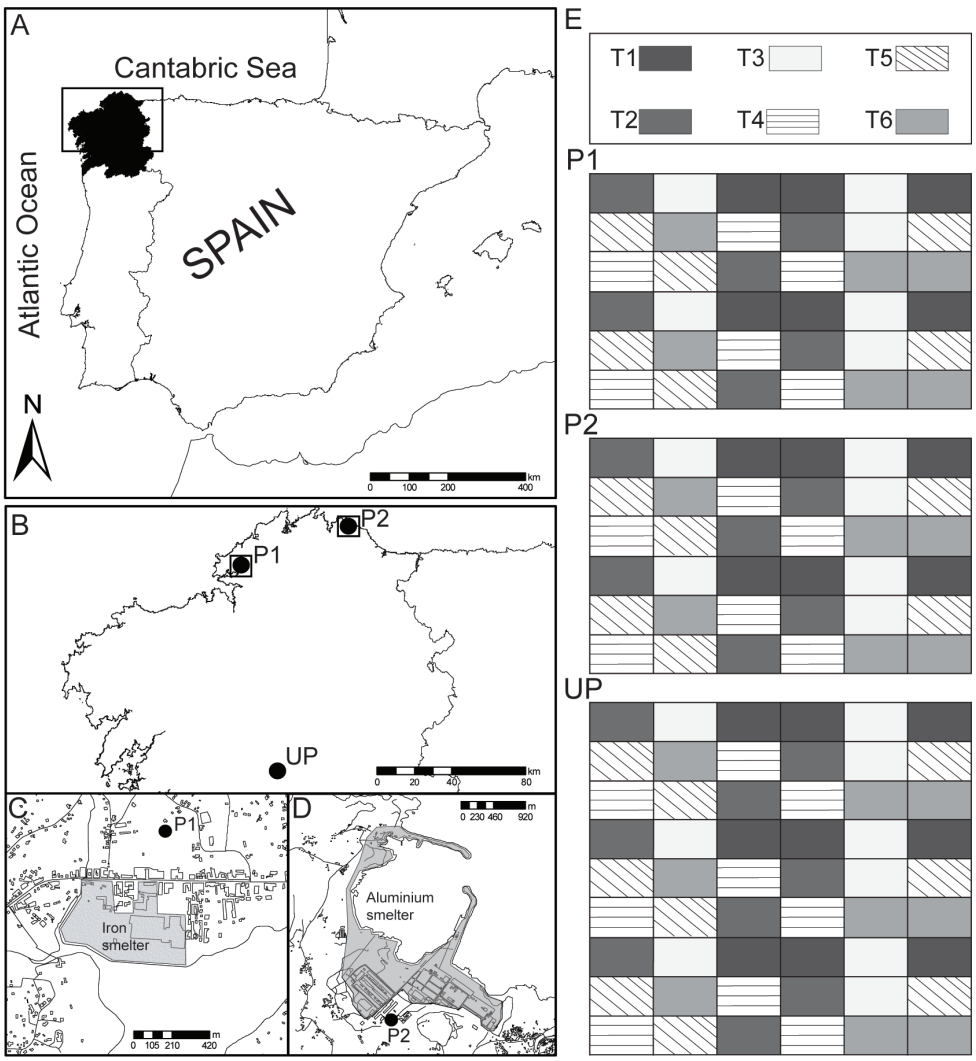


Fig. 1: Map showing the location of the sampling sites. A) Location of the study area in NW Spain; B) Location of the iron smelter (P1), aluminium smelter (P2) and unpolluted site (UP) within the study region; C and D) Details of P1 and P2, respectively, showing the source of pollution and the exact location of the sampling sites; E) Diagram of the experimental design used in sites P1, P2 and UP, respectively. For P1 and P2, the upper three rows of rectangles in each diagram represent the autotransplants and the lower three rows represent the transplants from the UP area. For UP, the upper three rows of rectangles represent the autotransplants, and the middle and lower three rows represent the transplants from P1 and P2, respectively (T1 to T6 represent the replicates transplants and autotransplants collected at each sampling period, from 1 to 6).

3. Results

The variations in the concentrations of the different heavy metals in moss samples transplanted from the UP sampling site to the P1 and P2 sampling sites and in moss samples autotransplanted within these sites are shown in Figs 2 and 3, respectively. The background levels of each metal, previously determined for Galicia (Carballeira et al., 2002), are also shown in the figures. The concentrations of all heavy metals in the moss samples moved from the UP site to either of the polluted sites tended to increase throughout the study period. Specifically, in the samples transferred to P1, the concentrations of metals increased, from approximately 6 times for Cu and Hg, up to more than 20 times for Pb and Zn, after 240 days. In the samples transferred to P2, the concentrations of Cd and Pb increased by up to 4 times in the same period (240 days). However, the concentrations of these elements in the moss samples from P1 and P2 transplanted in the UP site tended to decrease, to approximately the same levels as in the UP autotransplants. Decreases in the concentrations of metals of 1.2 times (Pb in samples from P2 transplanted in UP) and 5.7 times (Zn in samples from P1 transplanted in UP), relative to the initial concentrations, were detected after 240 days.

The values of the t-statistics and the p-values for the test comparing heavy metal concentrations in transplants and autotransplants in the three SS for the initial time (t=0) and the longest exposure period (840 days in the case of P2-UP comparisons and 480 days for P1-UP comparisons) are shown in Table 1. The data show that the initial concentrations of all heavy metals in UP differed significantly from those in P1 and P2 respectively, except for Zn in the UP-P2 comparison. In fact, those concentrations in autotransplants from UP are close to the background levels for each element during the entire period of study (see Figs. 2 and 3). In relation to the total exposure period (480 or 840 days), the differences were only significant in the following comparisons: concentrations of Hg in P1 autotransplants and the UP-P1 transplants; concentrations of Cd and Zn in P2 autotransplants and the UP-P1 transplants, and concentrations of Pb and Cd in the UP autotransplants

in and the P1 and P2–UP transplants.

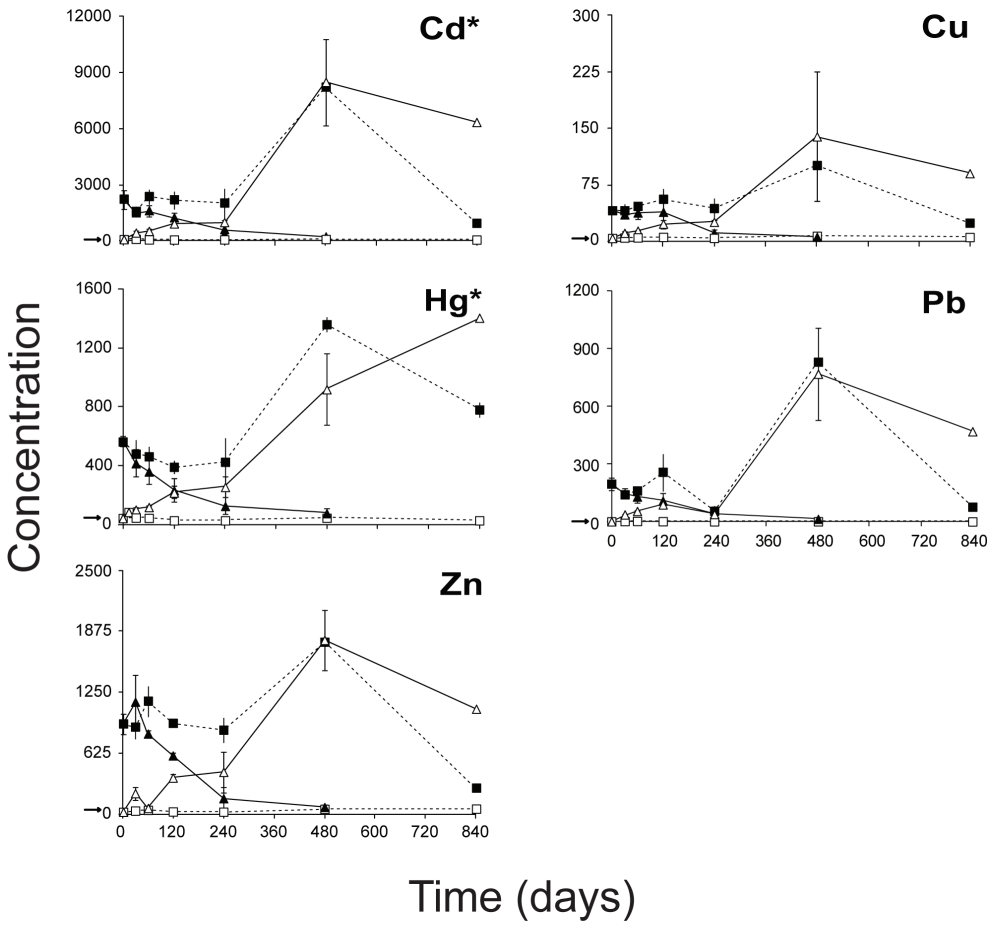


Fig. 2: Variation in the concentrations (μg·g^{-1}; *: ng·g^{-1}) of different heavy metals in the mosses transplanted between the unpolluted site (UP) and the iron smelter (P1) and in the autotransplants at P1, at the different sampling times. The error bars representing the 95% CI are also shown. Squares and continuous lines represent autotransplants; triangles and dashed lines represent transplants; open forms represent samples from the UP area; filled shapes represent samples from the P1 area; and the arrow (y axis) indicates the regional background level for each heavy metal.

4. Discussion

Concentrations of heavy metals in moss tissues are related to concentrations of these elements in the environment. However, moss concentrations depend on inputs and outputs of elements from different cellular compart-

ments: adhered particles, intercellular, extracellular, intracellular and intracellular particles. As the dynamics and equilibria of each of these are different, the concentrations that are finally obtained will be determined by the sum of all the processes. Moreover, the concentration in each compartment will be determined by the nature of the pollutant, the biological processes that occur within the moss, and environmental conditions. The compart-

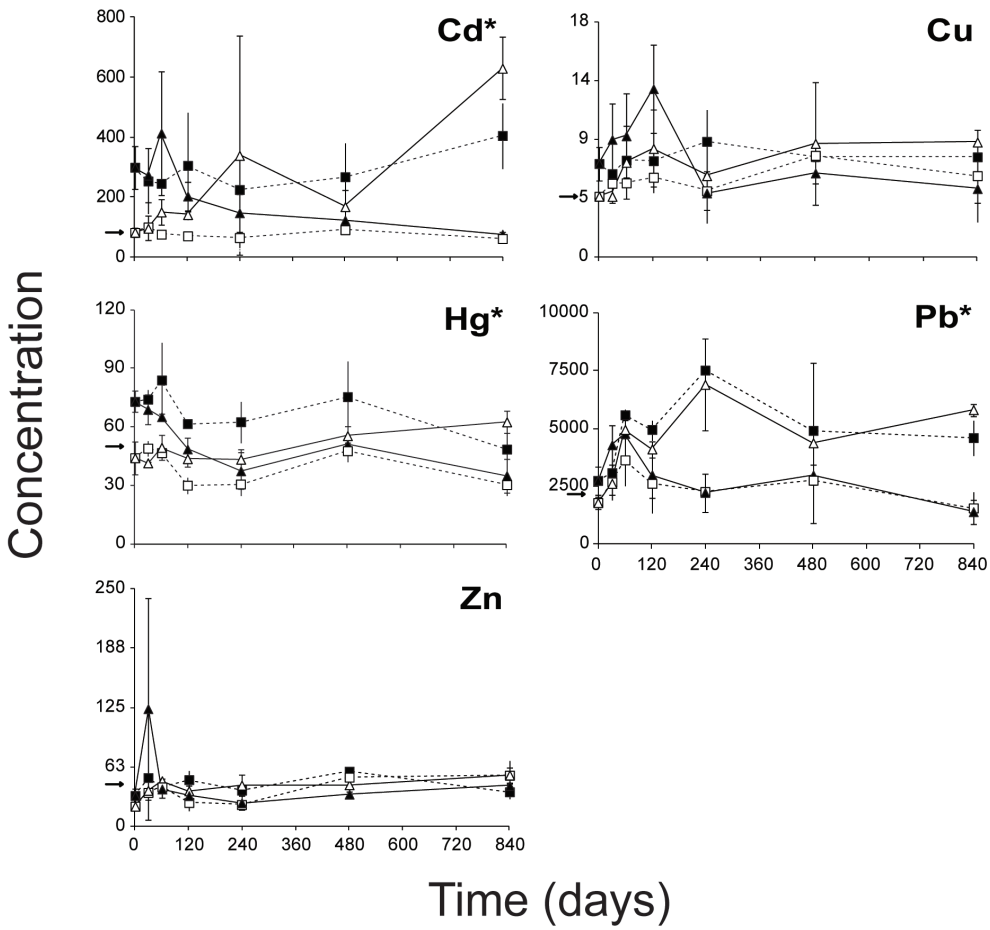


Fig. 3: Variation in the concentrations (μg·g^{-1}; *: ng·g^{-1}) of different heavy metals in mosses transplanted between the unpolluted site (UP) and the aluminium smelter (P2) and in the autotransplants at P2, at the different sampling times. The error bars representing the 95% CI are also shown. Squares and continuous lines represent autotransplants; triangles and dashed lines represent transplants; open shapes represent samples from the UP area; filled shapes represent samples from the P2 area; and the arrow (y axis) indicates the regional background level for each heavy metal.

ment in which heavy metals are located determine the toxic effects of the metals. Whether the response of the moss is to avoid or to overcome the toxicity, there will be a cost in terms of energy and/or biological efficacy, which in turn will affect the capacity of the organism to take up heavy metals.

4.1 Surface particulate matter

When particulate or other insoluble forms of contaminants are deposited on the moss surface, the concentration will depend on the following: i) the amount of airborne particulate matter that the moss receives; ii) the particle retention capacity; and iii) the release of attached particles. In the first place, the quantity and size of the particles present in the moss from the three SS is very different (Spagnuolo et al., submitted). The large amount of particulate material associated with the samples from P1 and P2 may explain the differences in the initial concentrations of all heavy metals in the samples from these sites and the UP site (Table 1), except for Zn between UP-P2. Secondly, the particle retention capacity of moss varies with the morphology and growth form. Characteristics such as the degree of gametophyte branching, density, surface area and degree of compaction of the phyllids

Table 1: Results of the two sample t-test comparing heavy metal concentrations in autotransplants at each sampling site and transplants between the sites (P1, P2 and UP), corresponding to the first (0 days) and last (480 or 840 days) sampling time. df: degrees of freedom; P1/P2-UP: comparisons of heavy metal concentrations in autotransplants from the P1 or P2 sites and transplants from the UP area; UP-P1/P2: comparison of heavy metal concentrations in autotransplants from the UP area and transplants from the P1 or P2 sites. (*: p<0.05; **: p<0.01; ***: p<0.001).

Element	df	P1-UP/UP-P1 0d	P1-UP 480d	UP-P1 480d	P2-UP/UP-P2 0d	P2-UP 840d	UP-P2 840d
Cd	4	8.34*	-1.231	1.932	5.759**	-2.825*	3.65*
Cu	4	16.326**	-1.072	-0.796	3.711*	-0.957	-0.063
Hg	4	29.083***	8.958**	1.428	5.362**	-2.508	0.498
Pb	4	11.397***	0.219	4.502*	3.695*	-3.423	-0.847
Zn	4	16.932**	-0.896	0.868	2.527	-3.154*	-1.294

and form of growth (i.e. isolated moss shoots compared with densely crowded shoots, or prostrate shoots compared with erect shoots) may also modify the concentrations of elements in moss due to changes in exposure to contaminants (Castello, 2007; Holy et al., 2009). The surface area of tissue in contact with the atmosphere (surface area/volume) will also determine the particle retention capacity of mosses (Carballeira et al., 2008). According to Vanderpoorten and Goffinet (2009), bryophytes display a wide range of intraspecific variation in their morphological characters depending on the environmental conditions under which they develop. This variation may generate a large amount of variability in the contaminant uptake capacity of specimens of the same species growing in different environments. In site P1, the concentrations of all heavy metals were higher in transplants from UP than in P1 autotransplants (see Fig. 2). Furthermore, in site P2, the concentrations of Cd and Zn were significantly higher in transplants from UP than in P2 autotransplants (Fig. 3 and Table 1). Although differences in concentrations of Hg and Pb were not significant (as for Cd and Zn), the error bars do not overlap. The lack of significance may be due to a lack of power of the test (due to the small number of replicates used, n=3) and, specifically for Pb, to a lack of equality of variance for the two samples being compared. These results may be explained by origin-dependent differences in the particle retention capacity of the moss, for the same levels of emission. Thus, in zones with high levels of atmospheric deposition, the retention capacity of the moss may decrease as an adaptive mechanism to minimize the uptake of contaminants.

Thirdly, precipitation may directly remove any particles adhered to the moss by mechanical action and, the absence of precipitation will decrease the hydration of the tissue, thus favouring the release of particles (Fernández et al., 2010). If this occurs, under equal conditions of emission, the concentrations of heavy metals in moss transplants from the contaminated SS will be expected to decrease rapidly to the same level as in the moss from the UP site. However, heavy metal levels in transplants from P1 and P2 only reached the same levels as in UP autotransplants at the end of the exper-

iment (except for Pb and Cd in transplants from P1 and P2 respectively) (Figs. 2 and 3 and Table 1). According to these results, we can assume that some of the particles are strongly retained in moss tissues (Spagnuolo et al., submitted), and indeed the final concentrations were only similar when new tissues grew under low levels of particle deposition, as in UP (transplants from P1 and P2 and autotransplants). The concentrations of heavy metals were determined in the apical segments (3-4 cm) of the moss shoots (see material and methods section). Taking into account that the annual growth of *P. purum* (in 2010) was 1.46 cm (unpublished data), the material analyzed after 840 days would consist almost entirely of new tissue (the ratio of new/ old tissue varied during the study period from almost 30% after 240 days to more than 50% after 480 days). It is possible that the concentrations of Cd and Pb in the moss from the contaminated SS did not reach the same levels as in the autotransplants (although the concentration of Cd reached the regional background level) because of errors in growth measurements and/ or variability in the size of the segments analyzed (which grew 3.36 cm in 840 days). Growth of the moss may also explain the length of time taken for the concentrations of heavy metals in the moss transplanted from UP to the contaminated SS to surpass the concentrations in the autotransplants (annual growth in P1 and P2 is 2.80 and 2.20 cm, respectively; unpublished data).

4.2 Intercellular and extracellular compartments

The extracellular concentration will depend on the following: i) the inputs of elements received by the moss wall and external layer of the plasma membrane; ii) the cation exchange capacity (CEC); and iii) the release of extracellularly bound metals. The percentage of extracellular metals is high in native *P. purum* in both of the contaminated sites: 73% of Hg and 54% of Zn in P1 (Pérez-Llamazares et al., 2009, 2011); and 49% of Cd and 77% of Zn in P2 (Fernández et al., 2013). However, these are probably greatly overestimated because metals may be extracted from particles attached to the moss surface during determination of the extracellular contents, as has been demonstrat-

ed for Zn in P1 (Pérez-Llamazares et al., 2011). It can therefore be assumed that the input of heavy metals to the extracellular compartment is less important than the input due to particulate matter.

Secondly, the uptake of soluble forms of pollutants (either in ionic form or derived from solubilization of the attached particles) will depend on the CEC of the moss. This is determined by the concentration and degree of methylation of uronic acids in the cell wall and outer layer of the plasma membrane (Richter and Dainty, 1989). Alterations in the concentration of galacturonic acids and in the activity of the enzyme catalyzing the methylation of these (Carballeira et al., 2008) and/or different morphological characteristics (Wells and Brown, 1987), may introduce intraspecific variability in the CEC of the cells and therefore in the uptake capacity of heavy metals. The CEC of the moss from the UP site may be higher, which would enable maximization of the uptake of nutrients in an environment with a low degree of deposition.

Thirdly, loss from the extracellular compartment may be due to various factors: particles being washed off by rain, displacement by cationic competition and anion complexing with the elements present in the solution, etc. Uptake from the extracellular compartment is rapid (in laboratory experiments uptake of heavy metals occurs within one hour) and varies over time (Fernández et al., 2013). If the contribution of the extracellular fraction to the total amount of heavy metals were high in this experiment, the changes in the concentrations over time would be much more rapid than those observed in Figs. 2 and 3. However, the results show a much slower decrease in the concentrations in the moss from P1 and P2 transplanted in UP, which may be explained by growth, as already indicated for the particulate matter, and by solubilization of the particles in the moss from the contaminated SS.

4.3 Intracellular compartment

Finally, heavy metals can reach the intracellular compartment as both soluble and insoluble forms. The intracellular concentration of heavy metals will depend on the following: i) the amount of elements in the intercellular and

the extracellular compartments; ii) transport of heavy metals to the interior of the cells; and iii) diffusion of heavy metals out of the cytoplasm. It is known that the contribution of heavy metals from the extracellular fraction is relatively less important than that of the intracellular fraction. Indeed, it has been demonstrated that there is no correlation between intra and extracellular concentrations of Zn and Cd in native *P. purum* in P2 (Fernández et al., in 2013).

Secondly, elements can be introduced in the cytoplasm of moss cells by passive (through channels) or active (mediated by primary or secondary transporters) transport (Raven et al., 1998), and therefore the regulation capacity of the uptake of elements will depend on the regulation of active transport. Although this type of transport has not been studied in detail in mosses, divalent cation membrane transporters with a wide range of substrates, have been found to be involved in hyperaccumulation of heavy metals in higher plants (Assuncao et al., 2001). In accordance with this, the existence of this type of transporter in moss may enable regulation of the active transport of micronutrients (e.g. Cu, Zn, Fe) along with that of other non essential cations (e.g. Cd, Pb) and will therefore enable the capacity for heavy metal uptake to be varied. Intracellular concentrations of some heavy metals (i.e. Cu and Zn) have been suggested to be regulated by specific membrane transport proteins (Basile et al., 2012; Lang and Weirnitzing, 2011). Inhibition of expression of genes that code the membrane transporters in the moss from the contaminated SS may explain why the moss from the UP site accumulated more heavy metals than the autotransplants from P1 and P2.

Thirdly, the possible movement of heavy metals out of the cytoplasm depends both on transport to the exterior and the amount of the element that has been rendered insoluble in the form of phosphates, mainly in the vacuoles (Bruns et al., 2001). The insoluble metal remained in the tissues of the moss from the contaminated SS and was only not detected in totally new tissue that had grown in an uncontaminated environment (Figs. 2 and 3), as speculated for the contaminants in the particulate and extracellular fractions.

To date, the only other study that has involved moss cross-transplants is a study by Tabors et al. (2004), who reported that the concentrations of heavy metals in moss transplanted from an uncontaminated area did not exceed those in native mosses, and also that the concentrations in moss transplanted from the contaminated area to the uncontaminated area remained almost constant throughout the entire experiment. The discrepancies between the present and the previous study may be due to the following: i) the earlier study did not use autotransplants (i.e. the concentrations in transplanted samples were compared with those in native mosses, possibly skewing the data), and ii) the heavy metals were measured in the same part of the moss in different years (the moss segment as it ages in the moss turf), which would eliminate the effect of growth in the final concentrations.

5. Conclusion

From the results of this study, we deduce that continuous exposure of native moss in P1 and P2 to high concentrations of heavy metals has altered some of the mechanisms and/or characteristics involved in the uptake and/or release of these elements in some cell compartments (e.g. morphological characteristics, concentration of galacturonic acids, and degree of methylation and amount and/or activity of cell membrane transporters). In addition, the concentrations finally determined in the moss tissues will also depend to a large extent on growth of the moss. The generation of new tissues under identical conditions of heavy metal emissions will minimize the intraspecific differences in the bioconcentration capacity of moss in uncontaminated environments (the levels of heavy metals in transplants and autotransplants will become more similar). By contrast, such differences will be enhanced in contaminated environments (in which the concentrations will be higher in mosses with a higher uptake capacity).

Hence, we can interpret this alteration as a genotypic adaptation (differences in final concentrations are maintained even when mosses of different provenances grow under identical atmospheric deposition) in the moss in order to overcome these adverse conditions. The final outcome of this re-

sponse will be that, after a long period of exposition (60 years in P1 and 33 years in P2), the mosses from P1 and P2 act as "excluders" rather than "good biomonitors" for heavy metals in this type of environment.

The implications of these results are that concentrations of heavy metals determined in mosses growing naturally in environments with high levels of heavy metal inputs will be lower than those in moss growing in environments that, a priori, would enable the theoretical accumulation capacity to be reached (taking into account their morphological and physiological characteristics). This precludes comparison of data obtained from moss growing in environments affected by different degrees of contamination, as the mosses will differ over the time in their capacity to take up contaminants. This considerably reduces the value of native moss as a bioindicator in industrial environments. Therefore, we recommend active biomonitoring, rather than passive biomonitoring, in this type of environment and also recommend further investigation on the mechanisms involved in this type of response.

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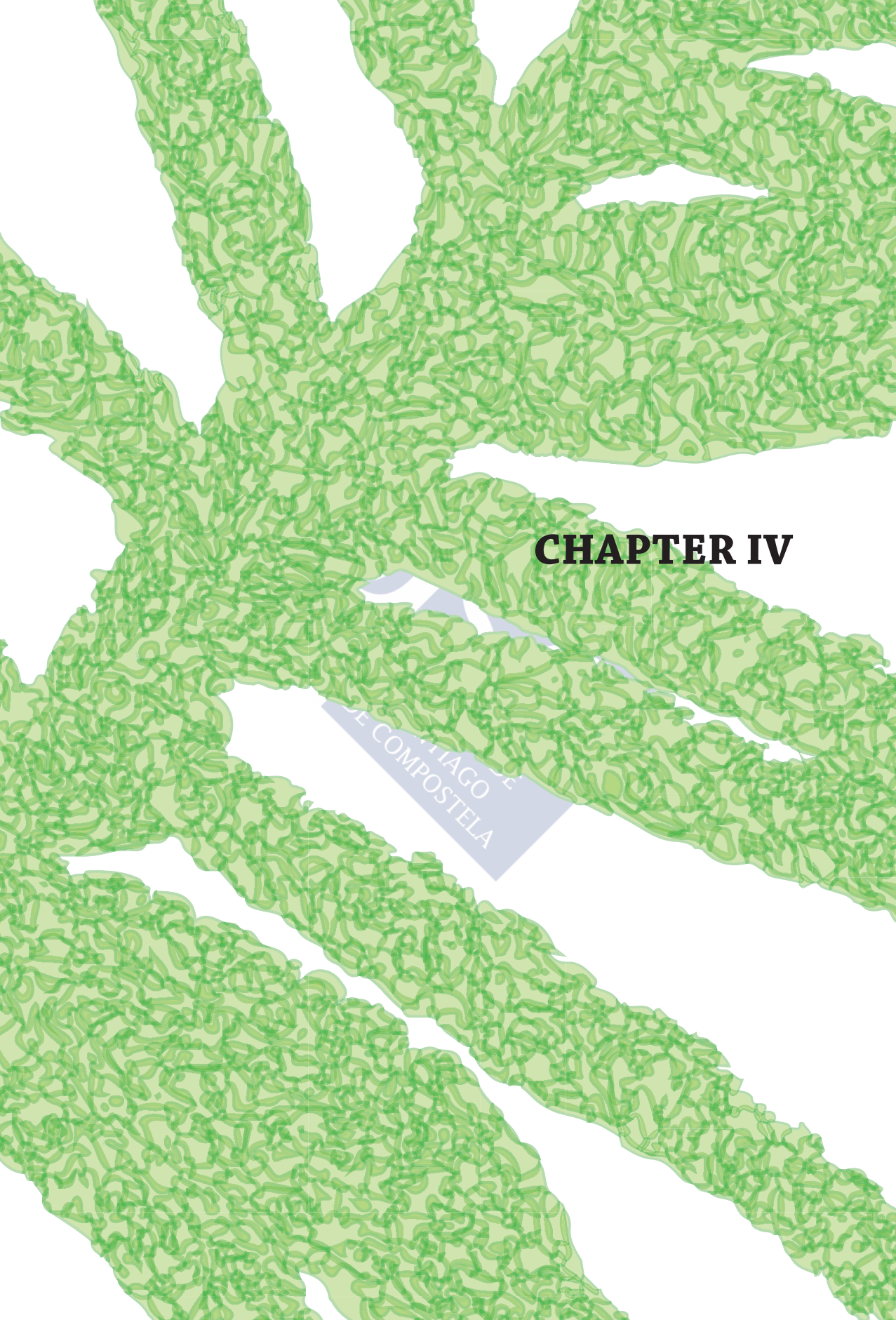
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CHAPTER IV

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Is it possible to estimate amospheric deposition of heavy metals by analysis of terrestrial mosses?

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Abstract

Here we present a critical review of diverse research studies involving estimation of atmospheric deposition of heavy metals from the concentrations of the contaminants in terrestrial moss. The findings can be summarized as follows: i) significant correlations between the concentrations of contaminants in moss and bulk deposition were observed in only 40.1% of the cases in which the relationship was studied and in only 14.1% of the cases, the coefficient of correlation was >0.7; ii) some method-related problems were identified (i.e. small sample sizes, elimination of some data from the regression analyses, large distances between the moss sampling sites and the bulk precipitation collectors, differences in times of exposure of the moss samples and collection times for the bulk precipitation), so that the results of the studies may not be completely valid, and iii) evidence was found in the relevant literature that moss does not actually integrate the atmospheric deposition received. We also discuss the reason why, in accordance with the published data, bulk deposition cannot be correctly estimated by determination of the final concentrations of contaminants in the organism, such as the existence of different sources of contamination, the physicochemical characteristics of the sources of deposition, physicochemical processes to which the organism is subjected and the biological processes that take place in the moss. Taking into account the above findings, it was concluded that, except for certain elements and specific cases (i.e. Pb and Cd), atmospheric deposition of elements cannot be accurately estimated from the concentrations of metals and metalloids in moss tissues. However, analysis of moss does provide information about the presence of contaminants in the atmosphere, their spatial and temporal patterns of distribution and how they are taken up by live organisms. Use of mosses is therefore recommended as a complementary (rather than an alternative) technique in the conventional analysis of bulk deposition of contaminants.

Keywords: *biomonitoring, bioindication, bryophytes, precipitation analysis*

1. Introduction

In the scientific literature heavy metal air pollution is evaluated either by measuring concentrations directly in the air, or more commonly, through the amount of them that are deposited on the earth's surface. Since the first investigation in which mosses were used as biomonitors (Ruling and Tyler, 1968), the moss technique has often been used to estimate atmospheric deposition of heavy metals. For example, Ross (1990) stated that: "(...) the use of mosses to estimate regional scale atmospheric trace metal deposition has gained acceptance"; similarly Berg et al. (1995a) pointed out that: "Use of mosses as a biomonitors is a well-established technique to estimate atmospheric trace element deposition on a geographical basis"; Berg and Steinnes (1997) also noted that: "Surveys of atmospheric deposition of metals, as reflected by their concentration in mosses (...)" and "The moss surveys describe regional differences and time trends in heavy metal deposition (...)". More recently, Harmens et al. (2008) maintained the same idea: "The technique of moss analysis provides a surrogate, time-integrated measure of the spatial patterns of heavy metal deposition from the atmosphere to terrestrial systems". As a consequence, the moss technique sometimes appears to be accepted as an alternative to quantification of the deposition by analysis of precipitation (Berg et al., 1995a).

These and other authors assume that the concentration of a substance in mosses is related to the atmospheric deposition of the substance. Two approaches have been developed in order to estimate the absolute deposition rates of metals by analysis of moss samples (Berg and Steinnes, 1997). The first is based on determination of the annual biomass production, assuming that the deposited metal is retained quantitatively in the top segment of the moss layer (Rühling et al., 1987), and differences in sorption efficiency are negligible although there are no empirical tests to support this hypothesis in mosses, but it was rejected in lichens (Bennett, 2008). The second, more widely used approach, is to calibrate the concentrations of elements in moss against bulk deposition values, obtained by analysis of precipitation sam-

ples, by use of linear regression (Berg and Steinnes, 1997; Berg et al., 1995a; Fowler et al., 2006; Ross, 1990; Schintu et al., 2005; Thöni et al., 1996). The regression equations obtained are then used to transform moss data to absolute deposition rates. In this article we will carry out a critical review of all these studies and the conclusions reached.

Many findings indicate that in general the concentration of a substance in moss cannot be closely related to its atmospheric deposition, so that it is impossible to use mosses to estimate the bulk deposition of metals as was previously suggested by Gombert et al. (2004). A series of biological and physicochemical processes, which take place after deposition of metal on the moss surface make this impossible. In the next section we will explain how we reached these conclusions from a brief literature review.

2. Evidence that the mosses do not integrate atmospheric deposition

Various authors (see for example: Berg et al., 1995a; Harmens et al., 2008; Ross, 1990) maintain that moss samples provide an integrated view of exposure over the corresponding period of apical growth. However this has not yet been verified, and there is some experimental evidence indicating that this idea is wrong. If mosses do not integrate bulk precipitation, we can deduce that it will be virtually impossible for the determination coefficients of the regression lines (concentrations in moss against atmospheric deposition) to reach values close to one. Furthermore, the same factors that indicate that moss samples do not integrate bulk precipitation will also prevent high determination coefficients being obtained for these regressions.

2.1. Comparisons between mosses exposed to bulk and dry deposition

In a comparison of auto irrigated shaded transplants of *Pseudoscleropodium purum*, Couto et al. (2004) found that in a high percentage of cases, the concentrations of metals and metalloids studied (Al, As, Cu, Fe, Hg, Mn, Ni, Pb and Se) were lower in transplants exposed to the bulk precipitation than in those exposed only to dry deposition, with the exception of Zn. As bulk deposition is the sum of dry and wet deposition, loss of metals was

demonstrated. These authors therefore concluded that moss does not integrate atmospheric deposition, but rather concentrates part of the deposition and remains in a state of unstable equilibrium between inputs and the outputs of elements, a state that is determined by the environmental characteristics (e.g. volume and pH of precipitation, concentration of pollutants, intercationic competition, etc.). Brown and Brūmelis (1996) have reported the existence of rapid re-equilibration to new environmental conditions in laboratory experiments, concluding that the frequently presented interpretation that mosses progressively accumulate elements throughout their life is false.

2.2. Short-time concentration variability studies

Laboratory experiments showed that very rapid element exchange occurred (Brown and Brūmelis, 1996); this would make mosses a less than ideal indicator of long-term pollution trends because element absorption will then have a reversible character. Mosses might then rather reflect the last rainfall instead of several years-accumulated depositions (Reimann et al., 1999), as a consequence mosses do not give a good reflection of the average chemistry of total average yearly precipitation.

Recently, Boquete et al. (submitted) studied the temporal variability in metal loads (i.e. Al, Cd, Cr, Cu, Fe, Hg, Ni, Pb, V and Zn) in shoot apices of the moss *Pseudoscleropodium purum* during the course of one year, with a high frequency of sampling (every 3 - 4 days). The results showed that the concentrations of the elements in the moss varied greatly within very short periods of time, with huge pulses of some metals. If the moss does integrate atmospheric deposition, the decreased contamination could only be explained by dilution as a result of growth. However the extent and pattern of growth was controlled in the study, so that the results could not be explained by growth-related dilution. The authors stated that the uptake of contaminants by moss is a complex process that depends on the nature and characteristics of the contaminants and moss species, and on the physicochemical and biological processes that affect the moss. All of these factors in

turn depend on environmental variables that may vary within a short time.

2.3. Intercalibration of metal concentrations between different moss species

In the vast literature on this subject (see for example: Fernández et al., 2000a; Halleraker et al., 1998; Ross, 1990; Thöni et al., 1996), low regression coefficients of determination (i.e. $r^2<0.65$) are often reported for inter-species comparisons (Carballeira et al., 2008). If mosses integrate atmospheric deposition, as the concentrations of both species are related to the independent variable (i.e. bulk precipitation), the coefficients of determination must be higher than usually found. Thus, the physiology and morphology of mosses are involved in the process of bioconcentration and passive uptake of contaminants from the atmosphere.

2.4. Internal redistribution of elements within the moss plant

According to Rühling (1994), because of the ability of mosses to integrate contaminants from atmospheric deposition, collection of annual segments of mosses enables assessment of the contaminants bioconcentrated by the moss throughout this period. However, several studies have shown that there may be movement of both nutrients and contaminants between the basal and apical parts of moss shoots (Brown and Brown, 1990; Brūmelis and Brown, 1997; Wells and Brown, 1996). Thus, it has been shown that the concentrations of nutrients such as K, Na and Mg are higher in apical parts (Bargagli et al., 1995; Grodzinska, 1978; Grodzinska et al., 1990; Tabors et al., 2004), while concentrations of metals such as Zn, Pb, Fe, Mn, Ba or Ni, are higher in the basal parts (Bargagli et al., 1994; Grodzinska, 1978; Grodzinska et al., 1990; Leblond et al., 2004; Pakarinen and Rinne, 1979). Fernández et al. (2010) have recently proposed an alternative hypothesis to explain the differences between sections of shoots. These authors suggest that differences can be explained by the involvement of active bioconcentration processes during growth of new moss tissues (i.e. particle entrapment, ion exchange, and passive intracellular uptake), in addition to passive processes. In any case it can be concluded that tissue concentrations of various ele-

ments in moss are not the result of a simple relationship between exposure and concentration, i.e. the moss does not really integrate the atmospheric deposition as the concentrations of elements in the tissues are not only due to this process.

2.5. Study of the relationship between concentrations of elements in moss and in artificial rain

Gjengedal and Steinnes (1990) studied the levels of accumulation of Zn, Cu, Cd and Pb in *Hylocomium splendens* and *Sphagnum fuscum* exposed to artificial rain solutions containing these elements. These authors concluded that bioconcentration processes are influenced by competition between and/or complexation of the major cations and anions present in precipitation. In addition, both the pH and temperature of the solution modify processes of heavy metal bioconcentration. Many studies have been carried out to investigate the efficiency of metal uptake from precipitation in mosses (see for example: Čeburnis and Valiulis, 1999; Čeburnis et al., 1999).

3. Calibration between moss data and atmospheric deposition

According to Reimann et al. (1999), a direct comparison of input (precipitation) and output signal (concentration in vegetation) should give a good indication whether or not a plant species is a suitable bioindicator. In the next sections we will review different studies focused in the relationship between precipitation and mosses.

3.1. Studies and results

We have had access to only six studies which have attempted to relate the concentrations of elements in moss and bulk precipitation in different geographical locations and with different species. The results of the regressions between the bulk precipitation and the moss data are shown in Table 1. Only 40% of all the regressions in these studies were significant ($p<0.05$); the percentages varied depending on the study: 69% in Ross (1990), 42% in Berg et al. (1995), 50% in Thöni et al. (1996), 26% in Berg and Steinnes (1997), 50% in Schintu et al. (2005), and finally 78% in Fowler et al. (2006). The determi-

nation coefficients obtained for the significant correlations were sometimes low (i.e. <0.7 in 65% of cases), so a large error may be introduced if bulk precipitation is estimated from the concentrations determined in mosses. There are differences in how each element behaves in comparison to the bulk deposition. If we study the results for those elements for which there are at least 5 data (Table 1), we can draw some conclusions: i) for some elements such as Cd and Pb the correlations were always significant (and for Pb for 50% of the data, r^2>0.9); ii) for other elements such as As, Fe, V and Zn, most of the correlations were significant (i.e. approximately 80%); iii) for others such as Cu, Mo and Ni, there was no definite pattern, and iv) for some elements the correlations were never significant (Mn), or only rarely (i.e. less than 20% of cases: Cr). With regard to Pb, the element for which the closest correlations between concentrations in moss and bulk precipitation were obtained, laboratory studies have shown that it is sorbed and retained to the greatest extent (Rühling and Tyler, 1970). It should be emphasized that little attention has been paid to Hg until now, despite it being highly toxic and able to be transported over long distances.

3.2. Problems identified in the moss-deposition calibration studies

Some of the results for the previous section should be considered with caution due to problems related to the methodologies used in the studies, as detailed below:

a) Number of samples: very few samples (n<10) were used in some of the studies, for example, Thöni et al. (1996) used only 3 for each element; Berg et al. (1995) used 6, and Ross (1989) and Schintu et al. (2005) used 8. The number of data only increased to 13 in the studies by Berg and Steinnes (1997) and Fowler et al. (2006).

b) Deleting data: although little data was available, in some studies approximately 15-20% (or unspecified amounts) of data were deleted during calculation of the regressions (e.g. Berg et al., 1995a; Ross, 1989; Berg and Steinnes, 1997; Fowler et al., 2006), without any clear criteria given for rejecting these data.

c) Distribution of bulk precipitation collectors: sampling stations are usually located in sites that are assumed to be unpolluted (e.g. Ross, 1989; Berg et al., 1995a) and few studies include sampling sites in contaminated areas (e.g. Berg and Steinnes, 1997; Schintu et al., 2005; Thöni et al., 1996). However, sometimes the data eliminated correspond to contaminated sites, e.g. 2 contaminated sampling stations in the study by Berg and Steinnes (1997). When performing regressions, the range of variation in the undetermined variable greatly affects the results. In general, if the range is narrow and only includes low values (below the level of the regional background), it is unlikely that significant correlations will be obtained. It is also important to consider how the data are distributed over the range, along with the available number of data, as these aspects may also generate problems. For example, in the study of Thöni et al. (1996), which considered only 3 samples, 2 of them almost always have very similar bulk precipitation values, so that almost all of the data are restricted to a single value. The regression lines must cross through only 2 points, which explains the unusually high percentage of significant correlations (i.e. 92%). Similar observations were made for some elements studied by Berg and Steinnes (1997).

d) Distance between collectors and moss sampling stations: the spatial variation in bulk precipitation is very high, and large distances between sites where mosses and bulk precipitation are collected may account for a high degree of error in the regressions. In the study by Berg et al. (1995), the distances are excessive, close to 50 km. The distances used in other studies are more acceptable, i.e. less than 1 km (Berg and Steinnes (1997) and Ross (1989), with exceptions of 4 km and 2 km at one sampling site in each study, respectively), less than 2 km (Schintu et al. 2005), and less than 0.5 km (Thöni et al., 1996).

e) Moss concentration data: the value of the dependent variable is not the same in all studies reviewed, and sometimes corresponds to the arithmetics mean concentrations of elements in a certain number of moss samples collected around the station where the bulk precipitation is collected

Table 1. Coefficients of determination and/or significance levels for the regressions between bulk deposition and moss concentrations by element, as reported in the available literature. r^{2sig}: coefficient of determination and significance level; ?: no coefficient available; a: Ross, 1990; b: Berg et al., 1995a; c: Thöni et al., 1996; d: Berg and Steinnes 1997; e: Schintu et al., 2005; f: Fowler et al., 2006; Hs: *Hylocomium splendens*; Ps: *Pleurozium schreberi*; Br: *Bryum radiculosum*; Hs-Ps: *H. splendens* and *P. schreberi*; and nPs: 5 species normalized by intercalibration with *P. schreberi*. n.s.: not significant, *: p<0.05, **: p<0.01

	r^{2sig}		r^{2sig}		r^{2sig}		r^{2sig}		r^{2sig}		r^{2sig}		r^{2sig}
Ag	? *(c,Ps)	Ce	>0.5**(d,Hs) ? *(c,Ps) ? n.s.(d,Ps)	Ga	? *(c,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)	Li	? *(c,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)	P	? n.s.(c,Ps)	Sn	? n.s.(c,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)	U	? *(c,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)
Al	? *(c,Ps)	Co	>0.5**(d,Hs) >0.5**(d,Ps) ? *(c,Ps)	Ge	? *(c,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)	Lu	? n.s.(d,Hs) ? n.s.(d,Ps)	Pb	0.99***(b,Hs) 0.98**(d,Hs) 0.98**(d,Ps) 0.93**(e,Br) 0.69***(f,nPs) 0.63*(a,Ps) 0.52*(a,Hs) ? *(c,Ps)	Sr	? n.s.(b,Hs) ? n.s.(c,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)	V	0.94**(d,Hs) 0.92**(d,Ps) 0.85**(b,Hs) 0.75**(a,Ps) 0.56*(a,Hs) 0.38*(f,nPs) ? *(c,Ps) ? n.s.(e,Br)
As	0.86**(d,Hs) 0.85**(d,Ps) 0.45*(f,nPs) ? *(c,Ps) 0.52n.s.(b,Hs)	Cr	? *(c,Ps) 0.03 n.s.(f,nPs) ? n.s.(a,Hs-Ps) ? n.s.(b,Hs) ? n.s.(d,Hs) ? n.s.(d,Ps) ? n.s.(e,Br)	Gd	? n.s.(d,Hs) ? n.s.(d,Ps)	Mg	0.82**(d,Ps) ? n.s.(b,Hs) ? n.s.(c,Ps) ? n.s.(d,Hs)	Pr	? n.s.(d,Hs) ? n.s.(d,Ps)	Ta	? n.s.(c,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)	W	? *(c,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)
B	? n.s.(c,Ps)	Cs	? n.s.(c,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)	Hf	? n.s.(c,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)	Mn	? n.s.(a,Hs-Ps) ? n.s.(b,Hs) ? n.s.(c,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)	Rb	? n.s.(b,Hs) ? n.s.(c,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)	Tb	? n.s.(d,Hs) ? n.s.(d,Ps)	Y	>0.5**(d,Hs) >0.5**(d,Ps) ? *(c,Ps)

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Ba	? n.s.(b,Hs) ? n.s.(c,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)	Cu	0.98**(b,Hs) 0.65*(a,Ps) 0.56*(a,Hs) ? *(c,Ps) 0.25 n.s.(f,nPs) ? n.s.(d,Hs) ? n.s.(d,Ps) ? n.s.(e,Br)	Hg	? *(c,Ps)	Mo	0.90**(d,Ps) 0.89**(d,Hs) ? *(c,Ps) 0.53n.s.(b,Hs)	S	? n.s.(c,Ps)	Te	? n.s.(c,Ps)	Yb	? n.s.(d,Hs) ? n.s.(d,Ps)
Be	? n.s.(c,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)	Dy	? n.s.(d,Hs) ? n.s.(d,Ps)	Ho	? n.s.(d,Hs) ? n.s.(d,Ps)	Na	? n.s.(b,Hs) ? n.s.(c,Ps)	Sb	0.95***(b,Hs) 0.95**(d,Hs) 0.88**(d,Ps) ? n.s.(c,Ps)	Th	? *(c,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)	Zn	0.93**(b,Hs) 0.75**(e,Br) 0.66*(a,Hs-Ps) 0.40*(f,nPs) ? *(c,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)
Bi	0.87**(b,Hs) ? n.s.(c,Ps)	Er	>0.5**(d,Hs) ? n.s.(d,Ps)	In	? n.s.(c,Ps)	Nb	? *(c,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)	Sc	? n.s.(c,Ps)	Ti	? *(c,Ps)	Zr	? n.s.(c,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)
Ca	? n.s.(c,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)	Eu	? n.s.(d,Hs) ? n.s.(d,Ps)	K	? n.s.(c,Ps)	Nd	? n.s.(d,Hs) ? n.s.(d,Ps)	Se	0.69**(d,Ps) 0.55**(d,Hs) 0.54**(f,nPs) ? n.s.(c,Ps)	Tl	0.94**(d,Hs) 0.64**(d,Ps) ? n.s.(c,Ps)		
Cd	0.84**(d,Hs) 0.82**(d,Ps) 0.82*(b,Hs) 0.73**(e,Br) 0.68*(a,Hs-Ps) 0.36*(f,nPs) ? *(c,Ps)	Fe	0.79**(a,Hs) 0.76** (a,Ps) >0.5**(d,Hs) >0.5**(d,Ps) ? *(c,Ps) ? n.s.(b,Hs)	La	? *(c,Ps) ? n.s.(b,Hs) ? n.s.(d,Hs) ? n.s.(d,Ps)	Ni	0.37*(f,nPs) ? *(c,Ps) ? *(b,Hs) ? n.s.(a,Hs) ? n.s.(a,Ps) ? n.s.(d,Hs) ? n.s.(d,Ps)	Sm	>0.5**(d,Hs) ? n.s.(d,Ps)	Tm	? n.s.(d,Hs) ? n.s.(d,Ps)		

–e.g. 5 in Berg et al. (1995), Thöni et al. (1996) and Berg and Steinnes (1997), and from 5 to 9 in Ross (1990) – whereas sometimes only a single moss sample was used. The values may also correspond to a mixture of species for some elements, as in Ross (1990), or to mean values for different intercalibrated species, as in Fowler et al. (2006). In the latter case the use of intercalibration equations with low coefficients of determination will cause a large error in subsequent correlations because using means results in a loss of information content.

f) Bulk precipitation data: the value of the independent variable is not the same in all studies and sometimes corresponds to the arithmetic mean values for a certain number of bulk precipitation collectors at each station (e.g. 5 in Thöni et al., 1996; 3 in Ross, 1990), although usually only a single collector is used. The bulk precipitation is sampled several times during the work (usually monthly). The multiple data are then processed to provide a single datum (usually expressed as a sum of the pollutant mass per unit area) that is correlated with moss concentration.

g) Duration of the study period: the length of the bulk precipitation data used in the regression analysis ranges from 1 year (e.g. Berg et al., 1995a; Thöni et al., 1996; Schintu et al. 2005), to 2 years (e.g. Ross, 1990; Berg and Steinnes, 1997). This may lead to invalid conclusions being drawn from comparisons of the corresponding results.

h) Length of periods during which bulk precipitation and moss data were collected: it seems obvious that the time that the moss has been exposed to atmospheric deposition should be the same as the time during which the bulk deposition was collected. When using *H. splendens* it is possible to determine the age of moss, and therefore the periods can be matched, as in Berg and Steinnes (1997). However, in other cases, even though the age of moss is known, these periods do not coincide, as in Berg et al. (1995) –3 years for moss and 1 year for bulk precipitation– or Ross (1989) –3 years for moss and 2 years for bulk precipitation. For other species (i.e. *Pleurozium screberi*, *Hypnum cupressiforme*, and *Bryum radiculo-*

sum) the green part of the shoots is used, regardless of the period that the section corresponds to, and without any method being used to quantify the growth (Bates, 1987). Only Fowler et al. (2006) used the length of shoot tips as an estimation of growth corresponding to one year for the 5 species studied.

After carrying out this literature review, and identifying these major problems, we consider that a properly conducted study relating moss concentrations and bulk deposition must meet the following criteria: i) more than 10 measurements should be obtained for each element; ii) data should not be deleted from the analysis; iii) a wide range of variation of bulk deposition should be included; iv) the distance between moss samples and collectors should be less than 2 km; v) the number of moss samples should be equal to or greater than three, for each collector; vi) the bulk precipitation should be quantified in at least 3 collectors, and vii) the measurement period for bulk precipitation and the exposure period considered for the moss must coincide. Only one study fulfilled as many as four of these criteria, while four studies met three criteria and the other study only complied with two of the criteria. Thus we can conclude that the number of studies is limited and the general quality of the studies is not high.

4. Reasons why bulk deposition should not be estimated from concentrations of elements in moss

We have clearly established that mosses do not integrate bulk deposition; in less than 15% of cases the determination coefficients for the regressions between concentrations of elements in moss and bulk precipitation concentrations were high (and taking into account the previously described problems, the proportion may be even lower in extreme cases). The underlying causes for these findings are complex, but briefly, the factors (which may act simultaneously) that make it difficult to estimate heavy metal deposition by analysis of terrestrial mosses are as follows: i) mosses have other inputs of elements in addition to airborne pollutants; ii) physicochemical characteristics of the deposition act along with physicochemical processes

that occur after metal deposition, and iii) different biological processes may act after deposition, thus modifying the final concentrations of pollutants in the mosses.

4.1. Types of inputs

De Caritat et al. (2001) reported that despite the conventional view that moss chemistry reflects atmospheric element inputs other sources of inputs (e.g. the nature of underlying mineral substrate) have a considerable influence on moss composition for several elements. Many studies have analysed these sources and the types of pollutants to which terrestrial mosses are exposed. These are usually identified by means of PCA analysis of metal concentrations in moss samples. In general, there are three different types of inputs: atmospheric, edaphic and from vegetation. We will briefly review each type.

a) Atmospheric inputs: anthropogenic sources, including long-range atmospheric transport or local point sources (Berg and Steinnes, 1997; Berg et al., 1995a; de Caritat et al., 2001; Reimann et al., 1999) of metals and metalloids such as: Ag, As, Bi, Cd, Co, Cu, Ga, Hg, Mo, Ni, Pb, Sb, Se, Sn, Tl, V and Zn. Such inputs also include natural sources such as sea salts and biogenic emissions from the marine environment: B, Br, Ca, Cl, I, Li, Mg, Na, S, Se, Sr and U. Other atmospheric inputs of elements are forest fires and volcanic emissions (Reimann et al., 1999).

b) Edaphic inputs: these correspond to mineral particles, mainly windblown soil, dust or transport of soluble compounds from the soil into the moss tissues during periods when there is close contact between soil and water (Berg and Steinnes, 1997). These inputs also may include substrate influence due to human activities, such as open pit mining, transport and storage of ore, and ore products (de Caritat et al., 2001). Sometimes moss chemistry appears largely dominated by input of local dust or airborne particular matter; it thus primarily reflects local site conditions and not major differences in atmospheric chemistry (Reimann et al., 2001, 2006; Steinnes, 1995). The elements mainly related to the edaphic inputs are:

Al, Co, Cr, Fe and Mn (see for example: Bargagli et al., 1995; Berg and Steinnes, 1997; Fernández and Carballeira, 2001). These inputs also include other elements such as: As, Ba, Ca, Ce, Co, Cu, Dy, Er, Eu, Ga, Gd, Ge, Hf, Ho, La, Li, Lu, Na, Nb, Nd, Ni, P, Pr, Rb, Sc, Si, Sm, Sr, Ta, Tb, Ti, Th, Tm, U, V, Yb, Y and Zr (Berg and Steinnes, 1997; Berg et al., 1995a; de Caritat et al., 2001; Reimann et al., 1999; Steinnes, 1995).

c) Plant inputs: these inputs have been identified by PCA analysis and also by correlations between metal concentrations in moss and the surrounding vegetation. In vascular plants, after uptake from the soil via roots, there is subsequent transfer to mosses by leaching from plant material (living or dead), mainly of Mn and Zn (Aboal et al., 2004; Berg and Steinnes, 1997; Berg et al., 1995b; Čeburnis and Steinnes, 2000) and also B, Ba, Ca, Cd, Cs, Cu, K, Mg, P, Rb and Sr (Berg and Steinnes, 1997; Berg et al., 1995b; de Caritat et al., 2001; Steinnes, 1995).

In light of the above comments it is clear that the existence of different sources of metals and metalloids for mosses has been known for two decades. However, if there are sources other than the atmosphere, mosses clearly cannot be used for accurate estimation of atmospheric deposition. It is evident that such estimation will be impossible for elements related to edaphic or plant sources (i.e. Al, Co, Cr, Fe, Mn and Zn).

However, it is the relative importance of each source that actually determines metal concentrations in moss, and if the atmospheric inputs are the main source, the estimation of bulk deposition from moss data would be possible. On the basis of the studies reviewed in section 3.1., elements such as Pb are available for the moss mainly from atmospheric deposition, but for others such as Cr (mainly edaphic input) and Mn (mainly vegetation input), this source makes little contribution to the composition of moss, which explains the absence of significant correlations between moss and deposition for both. Nevertheless, the relative importance of each input will depend on each moss sample; for example, in the surroundings of Cr or Mn production plants, mosses display high concentrations of both due to atmospheric inputs; in samples collected from areas with high edaphic concentrations of

Pb, the levels in mosses are hardly due to atmospheric deposition. This may explain why elements such as Al, Cr, Fe or Zn are still included in biomonitoring programmes (Harmens et al., 2008), although obviously the results must be considered carefully.

4.2. Physicochemical characteristics of pollutants and physicochemical processes in moss

Little attention has been paid to these aspects until now. The gaseous or particulate nature of pollutants will determine the form and proportion of each that will be accumulated in the moss. Likewise the chemical form of the pollutant will determine its solubility once the element is deposited. If metals are bound to particles or present in insoluble form, the uptake process only depends on the capability of the moss to retain particles. However, if the elements change to ionic forms, processes related to the cationic exchange capacity of the cell will determine the final concentration in moss; some authors have found differences in metal and metalloid uptake (Fernández et al., 2004).

Furthermore, the environmental conditions may alter the equilibrium between moss and deposition and the effects may differ at each location where mosses grow. The amount of precipitation may modify the solubility of the particles and also wash some of them away (Boquete et al., submitted; Couto et al., 2004). In the same way, the chemical composition of precipitation will play an important role in solubilisation of the particles, and also in displacing elements by intercationic competition (e.g. Cu or Zn) and anionic complexation (e.g. chlorides), thus avoiding uptake of metal cations (Gjendal and Steinnes, 1990). The pH of rain will be also a determining factor, as ion exchange with H^+ derived from acidic precipitation has been described (Gjendal and Steinnes, 1990). Finally, the distance from the sea may modify the cation content in the precipitation (i.e. Na, Sr and Mg), thus determining the final concentrations in moss due to ion exchange (Gjendal and Steinnes, 1990).

4.3. Biological processes in moss

The availability of the metals described above will not be the only factor determining the final metal concentrations in moss, as a series of biological processes are also involved. Firstly, mosses may enrich certain elements that they need for life function; some authors have reported low regional variation of the major nutrients that suggests that the uptake of these elements is regulated by the plant (see as example: Reimann et al., 2001). This regulation is only possible if there are specific carriers at the plasma membrane of cells (Brown and Bates, 1990). Unfortunately little attention has been given to other processes.

One of the biological variables that may modify moss metal contents is plant growth (Fernández et al., 2010). It is possible that metal uptake is not only proportional to the exposure time, but that during growth of the tissues, active processes (i.e. particle entrapment, ion exchange, and passive intracellular uptake) may take place, in addition to passive processes. This suggests a higher capacity for bioconcentration during the period of formation of new tissues, which may maximize capture of tissue constituents. Plant growth may also modify metal concentrations when the growth takes place after an episode of high uptake of pollutants. Nevertheless, if metal uptake is continuous, the dilution effect will not affect metal levels in moss, as this would be compensated by a larger surface area available for exposure. Changes in environmental conditions (e.g. season, climatic and microclimatic variables, etc.) may modify the moss growth strategies (growth rates would also vary due to genetic differences (Zechmeister et al., 2003) and determine its final concentrations (Halleraker et al., 1998; Zechmeister, 1995)). In the same way, the form of growth, not studied until now, may also modify concentrations of elements in moss due to changes in exposure to pollutants (i.e. isolated moss shoots compared with densely crowded shoots, or prostrate shoots compared with erect shoots) (Castello, 2007; Holy et al., 2009). Related to the growth, the age of the tissues also affects to the metal concentrations determined in moss (Brown and Brūmelis, 1996).

It is also possible that environmental variables (e.g. radiation, temperature, precipitation, etc.) may modify the uptake kinetics of metals in moss, also altering the final concentrations. For example, Brown and Brūmelis (1996) pointed out that desiccation stress can alter the cellular location of elements and then the total concentration. On the other hand, the precipitation type, as well as rain or snow, would also modify the metal uptake by mosses (Reimann et al., 1999).

Finally the existence in moss of mechanisms of adaptation to metallic pollution (phenotypic or genotypic) may reduce the concentrations of such metals in moss growing in polluted areas. In previous studies in which native and transplanted mosses are used simultaneously, the authors have hypothesized that differences in concentration in the different types of moss samples may be related to tolerance to metal pollution (Fernández and Carballeira, 2000; Fernández et al., 2000b). Recently, Tabors et al. (2004) used cross-transplantation between a polluted and non-polluted site and indicated that there appeared to be inherent differences in the ability of mosses from the different sites to absorb and accumulate heavy metals.

All the biological processes described above appear to be greatly affected by the characteristics of the environment where the moss grows. Differences in these characteristics in different zones, although all exposed to the same bulk deposition (amount and feature), will modify the final concentrations of elements in mosses, thus hindering estimation of the concentrations from moss data.

Final remarks and conclusions

The relationship between moss concentrations and bulk deposition is an important consideration and is the basis of the use of these organisms as biomonitors. However until now little effort has been devoted to studying this relationship and the topic is not always well handled by scientists working in this area. We believe that greater effort is needed to develop new research to enable robust and conclusive results on correlations between moss and bulk deposition.

In the research carried out to date, moss concentrations are sometimes significantly correlated with bulk deposition (40.1%), and in most cases cannot be predicted from the equations obtained because of the low determination coefficients (only 14.1% of cases with $r^2>0.7$). In light of current knowledge, the idea that terrestrial mosses can be used to estimate bulk deposition, as commonly assumed in almost all moss biomonitoring studies, should be abandoned. However, it appears that such estimations are sometimes possible for certain metals such as Pb or Cd, probably because these elements are of almost exclusively atmospheric origin. Nonetheless, additional efforts are required to understand the differences between these elements and others of mainly atmospheric sources.

Several previous studies have shown that mosses do not integrate metals from bulk deposition, but rather they remain in a state of unstable equilibrium between inputs and the outputs of elements, a state that is determined by environmental characteristics. The existence of sources of metal other than atmospheric deposition, differences related to the chemical form of deposited metals and physicochemical and biological processes may explain the lack of correlations between moss concentrations and bulk deposition.

Nevertheless, the lack of significant correlations for some elements does not mean that this organism is not a good biomonitor of air quality. Mosses provide information about the levels of bulk deposition and the temporal and spatial patterns of distribution, and also how this deposition interacts with a living organism through processes that depend on the environmental conditions in a particular study area. This information should be considered as complementary to conventional bulk deposition analysis, rather than as an alternative to the latter type of analysis. This also applies to other biomonitors such as lichens, forest litter, and needles and leaves from trees; indeed there are some evidences about this for lichens (Figueira et al. 2002), although they seem to be better integrators than mosses (Reimann et al., 1999), and epiphytic vascular species (Wannaz and Pignata, 2006).

Finally, we agree with Gombert el al. (2004) and Reimann et al. (2001) that the use of terrestrial mosses in metal biomonitoring enables spatial and

temporal comparisons (including regional biogeochemical mapping and detection of important element sources), as widely shown in the relevant literature. However, more attention should be given to interpretation of the results, which should not be considered as an absolute measure of metallic deposition, or a true reflection of atmospheric chemistry (Reimann et al., 2001), as this depends on the interaction between bulk deposition and environmental and biological variables.

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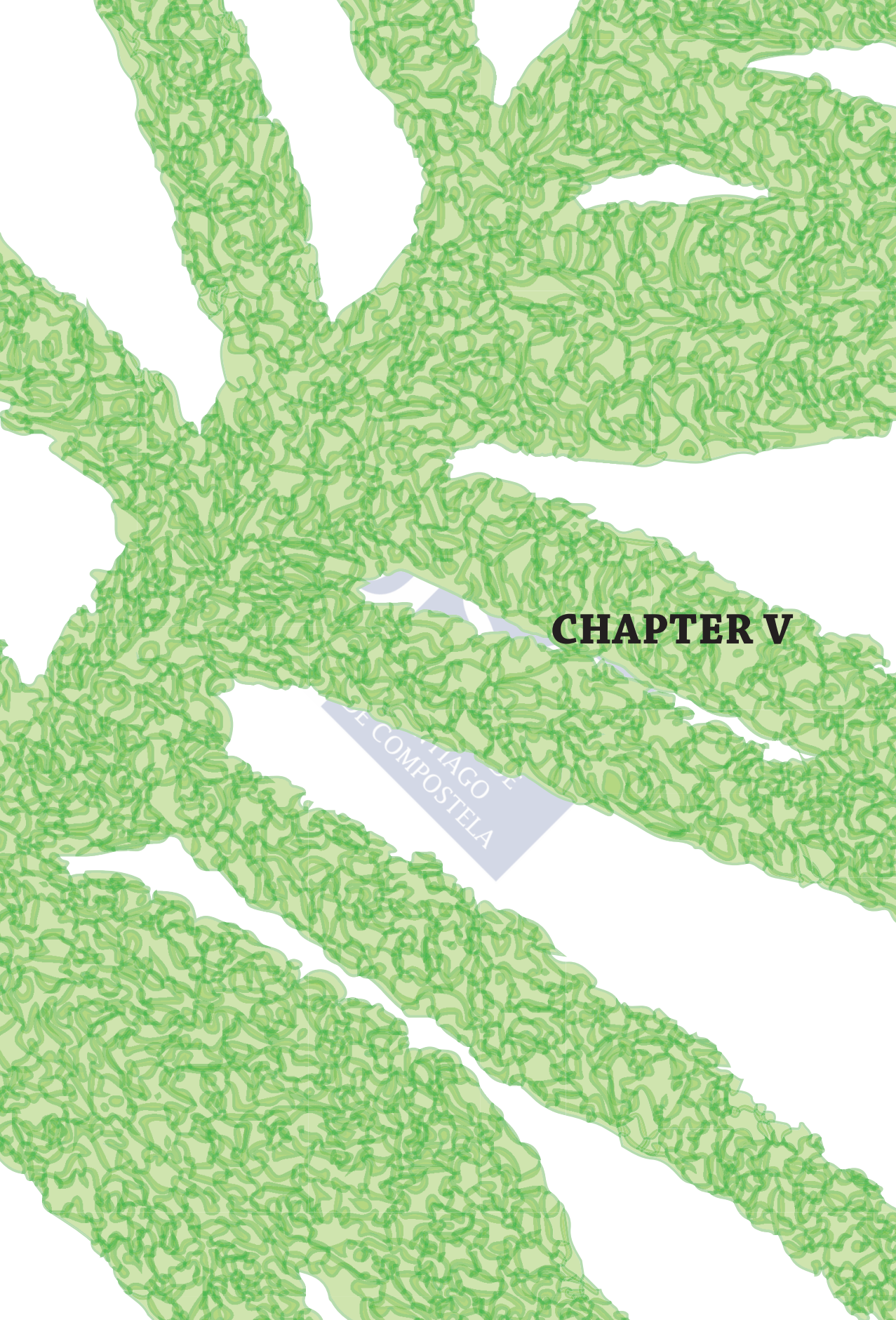
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CHAPTER V

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Are terrestrial mosses good biomonitors of atmospheric deposition of Mn?

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Abstract

A series of field and laboratory experiments was carried out to determine whether the terrestrial moss *Pseudoscleropodium purum* is a good biomonitor of the atmospheric deposition of Mn. Samples of the species were collected from sampling sites located in industrial areas and in unpolluted areas in Galicia (NW Spain). The results showed that: i) uptake of Mn by the moss may be reduced under certain conditions (related with the environment, moss ecophysiology and source and type of emission), even though atmospheric inputs and inputs from vegetation remain the same. The concentration of Mn in moss samples from the surroundings of an aluminium smelter was 6 times lower than in samples from an unpolluted site, in which levels were similar to the regional background level. The low concentrations were maintained over time; ii) the decrease in the uptake of Mn was due to differences in the environmental conditions to which the organisms are exposed. Genetic causes for the differences were ruled out as the study of moss samples transplanted within and between unpolluted and contaminated areas showed that the final concentrations in the transplanted moss in each of the scenarios were very similar; iii) the direct relationship between exposure and concentration was altered in the surroundings of the contaminated sampling site, although the causes of the inhibition of uptake could not be established by incubation of moss samples under laboratory conditions, and v) unlike other metals, there are few sources of emission of Mn to the atmosphere. However, when the emissions were in a particulate form, the concentrations of Mn in the moss varied in accordance with the atmospheric deposition. For all of these reasons, we consider that *P. purum* is not a good biomonitor of the atmospheric deposition of Mn, except under certain circumstances, and we recommend exclusion of the element from regional surveys.

Keywords: *terrestrial bryophytes, biomonitoring, atmospheric pollution, metals.*

1. Introduction

Terrestrial mosses have been used since the late 1960s (Rühling and Tyler, 1968, 1970) as biomonitors of air quality because of their physiological and morphological characteristics, which enable them to retain and/or bioconcentrate contaminants. A biomonitor can be defined as an organism (or part of an organism) that provides quantitative or semiquantitative information about the quality of the surrounding environment (Bargagli, 1998; Markert et al., 2003). There are two types of biomonitors: i) sensitive biomonitors, which provide information about the surrounding environment by means of measurable alterations in the organism itself (e.g. morphological, physiological or biochemical), and ii) accumulators, which provide such information after determination of the tissue concentrations of the contaminants taken up from the environment. The technique of biomonitoring air quality with terrestrial mosses is based on the capacity of the mosses to accumulate contaminants.

In recent decades, use of the technique has spread throughout the world, mainly because bryophytes capture most of the nutrients that they need directly from the atmosphere as they lack a complex system for internal regulation of the concentrations of the elements captured. Terrestrial mosses have therefore been classified as suitable for study of the atmospheric deposition of contaminants. However, it should be taken into account that for an organism to be classified as a "good biomonitor", it is not only essential that the organism can capture elements from the environment, but also that a simple linear relationship is maintained between the concentration of contaminants to which the moss is exposed and the concentration of contaminants in the organism, within the widest range of concentrations possible, and that capacity for uptake is not readily saturated. This is because only those organisms that meet this criterion will provide reliable information about the quality of the surrounding environment (Bargagli, 1998). To be a good biomonitor the organism must conserve the levels of the contaminant taken up, i.e. it must not regulate the levels of the element.

However, sufficient attention is not usually given to the fact that the concentration of contaminants in the moss does not only depend on the inputs of contaminants, but also on factors such as the physicochemical characteristics, physicochemical and/or biological processes, and the environmental variables to which the organisms are exposed. These complex processes may alter the linearity of the relationship between exposure and concentration and lead to a lack of any relationship between the concentration of diverse elements in the moss and the bulk deposition, described on different occasions by various authors (Ross, 1990; Berg et al., 1995b; Thöni et al., 1996; Berg and Steinnes, 1997; Čeburnis and Steinnes, 2000). Of all the elements studied, Mn is the only one for which there was no significant correlation between the concentrations in moss and in the bulk deposition, so that Mn is the element that best illustrates the problem of the lack of linearity in the relationship between exposure and concentration in biomonitoring studies with terrestrial mosses. Among all of these works, in the studies made by Ross (1990) and Ceburnis et al. (1999) it has been proposed the exclusion of Mn from the regional surveys. These studies have hypothesized which causes can be responsible of the results obtained without doing any kind of experiment to check them.

As a result, until now there is still a lack of knowledge about process related with Mn bioconcentration in moss tissues. In this way, the lack of specific works that have studied this topic in a direct form have prevented that the results presented until now have not been conclusive enough to completely exclude Mn from biomonitoring studies with terrestrial mosses. On the other hand, we have not found any study in which the factors which cause these results have been investigated. In fact we have found tens of articles which include Mn as one of the studied pollutants. For all of these reasons, in this work a series of field and laboratory experiments have been carried out in order to determine if terrestrial mosses are good biomonitors of the atmospheric deposition of Mn and if not, to check some possible causes of this. More specifically, the experiments were carried out to determine: i) if the uptake of Mn may be decreased under certain environmental condi-

tions leading to a lack of linearity in the relationship between exposure and concentration; ii) if such a decrease in uptake is maintained over time; iii) if the reasons for the decrease in uptake of Mn are due to the environment or to genetic adaptations of the moss to the environment; iv) if the concentrations of F deposited or the composition of the bulk precipitation are the causes of the decrease in the uptake of Mn by the moss, and to demonstrate v) that the concentration of Mn does not usually increase in the tissues of moss growing in the areas surrounding focal points of industrial Mn contamination.

2. Material and Methods

2.1 Sampling and processing

In all cases, samples of the terrestrial moss *Pseudoscleropodium purum* (Hedw.) M. Fleisch. were collected at different sampling sites (SS) located in Galicia (NW Spain) and sampled on different dates.

2.1.1 Spatial relationship between the concentrations of Mn and other metals in the surroundings of a focal point of contamination

With the aim of determining if the uptake of Mn decreases under certain environmental conditions, thus causing the absence of a linear relationship between exposure and concentration, samples of moss were collected, in November 1998 and May 1999, from 50 SS located in the surroundings of an aluminium smelter that emits several heavy metals and metalloids (Fernández et al., 2007), and, above all F (Real et al., 2003). Details of the sampling and processing methods are provided by Real et al. (2003).

2.1.2 Temporal changes in the concentrations of Mn in the moss tissues

With the aim of determining if the decrease in the uptake of Mn only occurs at particular times or if it is prolonged over time, high frequency sampling was carried out, during 2005, in the surroundings of the above-mentioned factory and in an unpolluted area. At both sites, the samples were collected twice weekly (i.e. every 3 or 4 days) during the entire sampling period. In the

surroundings of the aluminium smelter, the sampling began on 3rd January and continued until 29th December (n = 101). In the unpolluted area (Aboal et al., 2004), the sampling began on 29th May and ended on 29th December (n=62). Details of the sampling and processing methods are provided by Boquete et al. (submitted).

2.1.3 Changes in the concentrations of Mn in the moss transplanted between contaminated and unpolluted areas

To determine if the decrease in uptake of Mn was due to environmental causes or to genetic adaptations of the moss to a particular environment, a moss transplant experiment was carried out, between 2008 and 2009, with the moss *P. purum*. Samples of the moss were transferred from an unpolluted SS in the surroundings of the previously mentioned aluminium smelter and viceversa. Some moss samples were also transplanted within each of the SS. The transplants were carried out by separating a small portion of moss mats (approx. 15 x 15 cm) within a frame of height 3 cm (Cenci, 1998), and placing these in holes of the same size as the frames. Three replicate transplants were carried out at each SS, and were collected after exposure periods of 1, 2, 4, 8 and 16 months.

Prior to their analysis, the samples were cleaned, and apical segments (3-4 cm length) were cut from the shoots; all material adhering to the segments was removed. The samples were maintained in a moisture-saturated atmosphere at 10ºC for one week to ensure integrity of the membranes. The samples were then washed in bidistilled water, with shaking, for 30 seconds.

2.1.4 Uptake of Mn in moss under laboratory conditions

To determine the causes of the decrease in the uptake of Mn (e.g. the concentrations of F in the deposition or the composition of the bulk precipitation), samples of moss were collected at a SS located in a rural area, distant from urban and industrial areas. The moss was maintained in a moisture saturated atmosphere at 10ºC for one week and was then washed in bidistilled water, with shaking, for 30 minutes, with changes of water every 10

minutes. Three replicates each of 6 g of moss were then separated for determining the reference value of the concentrations of Mn in the moss under field conditions; the aliquots of moss (6g) were incubated with shaking for one hour, in the following solutions (with three replicates for each treatment): i) two samples of bulk precipitation collected, via a polythene funnel connected to a flask of the same material, at two SS, one unpolluted and the other in the surroundings of the aluminium smelter; ii) three solutions containing 0.5, 2 and 5 $\mu g \cdot ml^{-1}$ Mn, obtained from $MnSO_4$; iii) three solutions with the same concentrations of Mn and a fixed concentration of 10,000 $\mu g \cdot ml^{-1}$ F, and 12,130 $\mu g \cdot ml^{-1}$ Na, obtained from FNa. Fluorine was used because it is abundant in the surroundings of the aluminium smelter, and it was suggested that it may have an effect on the uptake of Mn by moss in the area; iv) three solutions with the same concentrations of Mn and the same concentration of Na, obtained from Na_2SO_4. As the solutions of F were prepared from FNa, Na solutions were used separately to study the possible existence of a process of displacement of Mn from the cation exchange sites on the moss, and to distinguish their effect from that caused by F. At the end of the incubation period, the samples of moss in the different solutions were washed in 2 L of bidistilled water. The samples were then dried in an oven at 45 ºC.

2.1.5 Determination of the existence of small scale focal points of contamination by Mn

In order to demonstrate if industrial focal points of contamination do not usually produce increases in the concentrations of Mn in their surroundings (because the element is not emitted or because the moss does not have the capacity to bioconcentrate the element), samples of moss were collected, some on different occasions, from the surroundings of 37 different production plants associated with the industrial sectors in the study region (ceramic industry, cogeneration in food industries, iron and other metal smelters, production of chlor-alkali, paper, bioethanol, cement-lime, glues, Mg compounds and glass, aluminium transformation, and treatment of used oil and

of solid urban waste). The method of detecting small scale focal points of contamination proposed by Fernández et al. (2007) was used for this purpose. Sampling and sample processing were carried out in accordance with the methods described by these authors.

2.2 Chemical analysis

The samples were homogenenized in an ultracentrifugal mill (Retsch ZM 100 or Retsch ZM 200 metal-free), then digested with HNO_3 (65% for analysis) in a microwave oven (CEM MDS2100), except in experiments 2.1.3 and 2.1.4, in which ultrasound extraction was used.

The concentrations of Mn were determined by flame atomic absorption spectrophotometry (Perkin Elmer 2100), except for the samples in experiment 2.1.5 and some in experiment 2.1.3, which were determined by graphite furnace atomic absorption spectrometry (Perkin Elmer AAnalyst 600). The concentrations of Mn in the bulk precipitation samples were determined by ICP-MS (VARIAN 820-MS). In experiments 2.1.1 and 2.1.5, the concentrations of Al, As, Cr, Fe, Ni and V and of Al, As, Ba, Be, Cd, Co, Cr, Cu, Fe, Ni, Pb, Se, Sr, V and Zn, were measured by flame atomic absorption spectrometry (Perkin Elmer 2100) or, when the concentrations did not reach the limits of quantification, by graphite furnace atomic adsorption spectrometry (Perkin Elmer AAnalyst 600). The concentrations of Hg were determined in an elemental analyzer (Milestone DMA80).

To ensure the analytical quality of the determination of Mn, two replicates of the same sample were analyzed every nine samples. In addition, four certified reference materials were analyzed: Poplar (GBW07604 poplar leaves; Institute of Geophysical and Geochemical Exploration, Langfang, China), M2 and M3 (*Pleurozium schreberi*; Steinnes et al., 1997) and an internal reference material (*Pseudoscleropodium purum*), also once every nine samples. To control for any possible contamination, analytical blanks were also included, once every nine samples. In general, the analytical quality of the process was satisfactory. For Mn, the overall percentage of error (Cêburnis and Steinnes, 2000) ranged between 2 and 9%, and the percentage recovery,

between 87 and 118%. In all cases, the concentrations obtained were above the limits of quantification (LQ) of Mn in the different apparatuses.

2.3 Statistical analysis

In order to analyze the existence of small scale focal points of contamination, we first calculated the probability that the differences in concentration between the pairs of moss collected around the focal points of contamination belonged to the distributions of the concentrations of different elements in unpolluted areas, as described by Fernández et al. (2007). Secondly, a Mann-Whitney U test was used to test the existence of differences between the concentrations of Mn in the samples of moss subjected to different treatments, by use of SPSS (version 15.0).

3. Results

3.1 Spatial relationship between the concentrations of Mn and other metals in the surroundings of a focal point of contamination

The spatial distribution of the mean concentrations (from the two sampling surveys) of Mn in the surroundings of the aluminium smelter is shown, along with the spatial distribution of the concentrations of the other metals and metalloids determined, in Fig. 1. The SS in which the range of concentrations of the different elements in moss are below or include the regional background level for each element (Carballeira et al., 2002, for heavy metals and metalloids; Real et al., 2003, for F) are shown in black. It can be observed how the concentrations of Mn and of all other elements determined, are similar to their respective regional background levels in the SS furthest from the aluminium smelter. However, the pattern observed for Mn was the opposite of that observed for the other elements; although the concentrations of all the other elements were well above their respective regional background levels in the SS closest to the aluminium smelter, and decreased at the SS furthest from the smelter, the concentrations of Mn were well below the regional background level in the SS closest to the aluminium smelter, but increased to levels close to the background levels in

the SS furthest from the smelter. Although Na and Mg are not showed in Fig. 1, we checked that there was not any significant regression between concentrations of both elements in moss (y) and the distance to the sea (x)

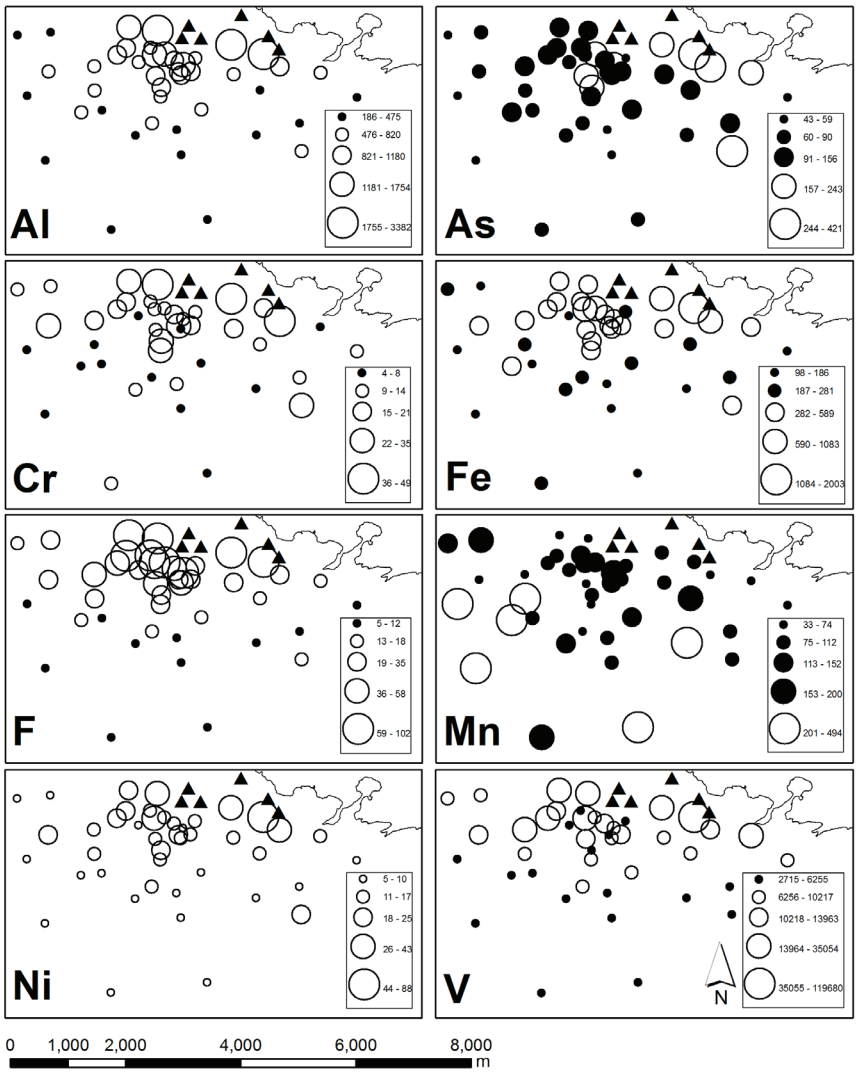


Fig. 1. Spatial distribution of the concentrations of metals and metalloids in the surroundings of an aluminium smelter. The size of the circles indicates the concentration shown in the corresponding legends (Al, Cr, Fe, F, Mn and Ni, $\mu g \cdot g^{-1}$; As and V, $ng \cdot g^{-1}$). The black-filled circles represent the SS in which the range of concentrations of the different elements in the moss are below, or include the value of the regional background level of each element. The triangles represent the different focal points of emission from the installation.

of each sampling site (n=87; r^2=0.0037 and r^2=0.0001, respectively); the slopes not differed significantly from 0 (p=0.578 and p=0.914, respectively). In addition, there were not significant regressions between Mn (y) and Na or Mg concentrations in moss (x) of each sampling site (n=87; r^2=2·10-5 and r^2=0.002, respectively); the slopes not differed significantly from 0 (p=0.970 and p=0.695, respectively).

3.2 Temporal changes in the concentrations of Mn in moss tissues

To examine whether the previous finding was maintained over time and was different from that in other sites not adjacent to the aluminium smelter, the tissue concentrations of Mn in moss samples from the unpolluted SS and from the surroundings of the aluminium smelter, and the regional background level for Mn in the study area (Carballeira et al., 2002) are shown for both washed samples (Fig. 2a) and unwashed samples (Fig. 2b). The results show the low uptake of Mn remained constant over time in the surroundings of the aluminium smelter. The figure illustrates how the concentrations of Mn in the samples from the surroundings of the alumunium smelter remained extremely low, below the levels of Mn observed in a clean SS (similar to the regional background level, 171 $\mu g \cdot g^{-1}$).

3.3 Changes in the concentrations of Mn in moss transplanted in a contaminated and unpolluted zone

In order to determine whether the decrease in the uptake of Mn is caused by environmental factors, or to genetic adaptations of the moss to the environment, samples of *P. purum* were transplanted within and between different environments. The changes in the concentrations of Mn in the samples are shown in Fig. 3. The concentration of Mn in the moss samples transferred from the unpolluted SS to the surroundings of the aluminium smelter decreased by more than 700 $\mu g \cdot g^{-1}$ in only 4 months, reaching a level lower than the regional background level in less than a year and a half. However, the concentration of this element in the moss samples transferred to the unpolluted SS increased by 12 times in only 8 months. It can also been seen

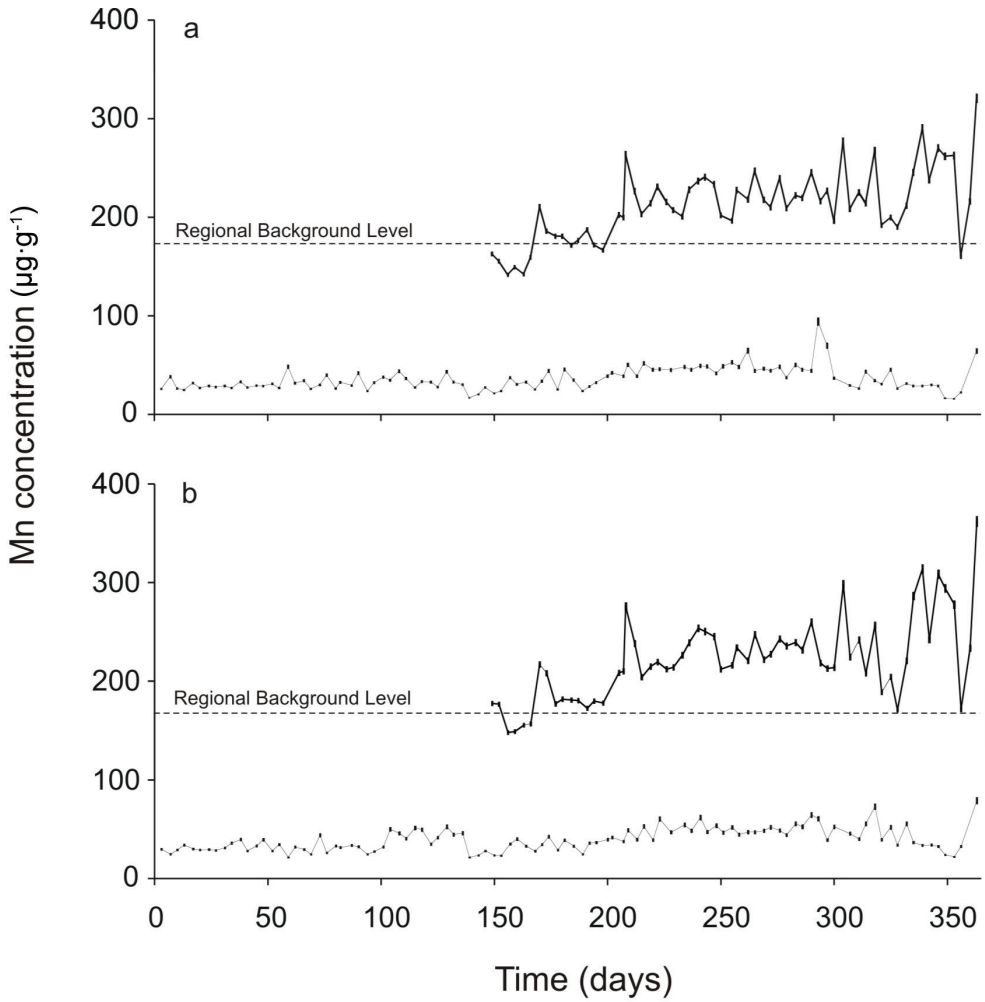


Fig. 2. Changes in the concentrations ($\mu g \cdot g^{-1}$) of Mn in the samples of (a) washed moss and (b) unwashed moss over time. The error bars represent the error associated with the analytical determination. Grey line: samples from the polluted area (aluminium smelter); black line: samples from the unpolluted area; dashed line: regional background level of Mn.

that the concentration of Mn in the samples transplanted within the contaminated area were maintained well below the regional background level for this element throughout the period of sampling.

3.4 Uptake of Mn in moss in laboratory experiments

The mean values (and the 95% confidence intervals) of the concentrations of Mn in the samples of moss subjected to different treatments are shown

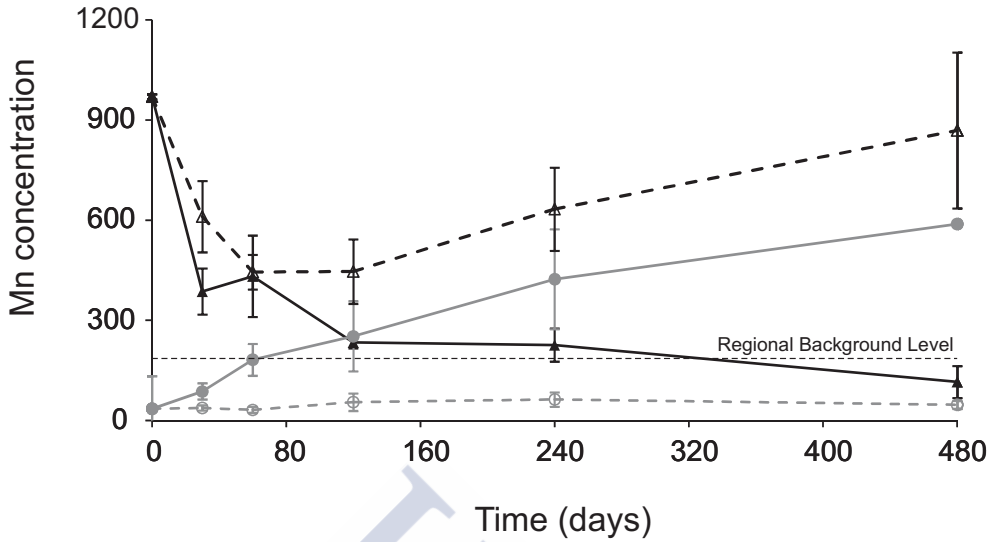


Fig. 3. Changes in the concentrations (μg·g^{-1}) of Mn over time in the samples of moss transplanted within and between different areas. Grey lines: samples from the polluted area (aluminium smelter); black lines: samples from the unpolluted area; continuous lines: transplanted samples (polluted to unpolluted and unpolluted to polluted); dotted lines: samples transplanted within the same area; thin dashed line: regional background level of Mn.

in Table 1. The data in this table demonstrate an increase in the concentration of Mn with increasing concentration of Mn in the incubation solution. In accordance with the results of the Mann-Whitney U test, there were no significant differences ($p<0.05$) between the concentrations of Mn in the samples of moss subjected to the 3 different incubations for each of the concentrations of Mn in the solution. With regard to the samples of moss incubated in the bulk precipitation collected in the surroundings of the aluminium smelter (13.7 μg·L^{-1} de Mn) and the unpolluted SS (6.93 μg·L^{-1} of Mn), the concentrations of Mn reached in the moss were 381±10 and 387±41 μg·g^{-1} (mean ± 95% confidence interval), respectively. The Mann-Whitney U did not reveal any significant differences in the concentrations of Mn in the moss incubated in these bulk precipitation samples.

3.5 Determination of the existence of small scale focal points of Mn contamination

The behaviour of Mn was completely different from that of the other 17

Table 1. Mean and the corresponding confidence interval (95%) of Mn concentrations in moss tissues (μg·g^{-1}), obtained after incubation in different solutions: i) $MnSO_4$; ii) $MnSO_4$+FNa; and iii) $MnSO_4$+$NaSO_4$, in which the concentrations $MnSO_4$ were varied (0, 0.5, 2 and 5 μg·ml^{-1}). The concentration of F in the incubation with $MnSO_4$+FNa was 10,000 μg·ml^{-1} and the concentration of Na in the incubation with $MnSO_4$+FNa and $MnSO_4$+Na_2SO_4, 12,130 μg·ml^{-1}.

μg·ml^{-1} Mn in solution	Mn (μg g^{-1}) in moss tissues								
	$MnSO_4$ incubation			$MnSO_4$ + FNa incubation			$MnSO_4$+Na_2SO_4 incubation		
0	502	±	31	547	±	18	386	±	22
0.5	1230	±	70	1003	±	54	770	±	47
2	2483	±	88	2276	±	246	1749	±	59
5	3635	±	180	2831	±	189	2911	±	87

metals and metalloids studied in the same factories, as of the 50 cases studied (37 factories, some sampled on more than one occasion), contamination by Al, Ba, Co, Fe, Ni, Pb and V was detected in more than 50% (the latter in 74% of cases), contamination by Be, Cd, Cr, Cu, Hg, Sr and Zn was detected in 30% of cases, and contamination by As and Se in 20% of cases, and by Mn in only 4% of cases. However, when contamination by Mn was detected (in November 2006 and March 2007) in the surroundings of an Fe-Mn smelter, it was very clear. In both sampling surveys, the concentrations of Mn in samples from sites close to the factory were higher than 2,000 μg·g^{-1} n (2,046 and 2,983 μg·g^{-1} in 2006 and 2007, respectively), but decreased to below 250 μg·g^{-1} at a distance of some km.

4. Discussion

The results of the study of the spatial distribution of the concentrations of Mn and other metals in the surroundings of the aluminium smelter show that the concentrations of Mn increased with the distance from the smelter, from around 33 μg·g^{-1} Mn to between 200 and 500 μg·g^{-1} Mn in samples from the most distant SS (Fig. 1). Considering that, as already mentioned, most of the concentrations of Mn registered close to the factory are well below the regional background level of Mn (171 μg·g^{-1}), there may be some factor related to the surroundings of the factory that cause the low concen-

trations of Mn in the moss. The low concentrations were maintained over time so that the mean concentration of Mn throughout the year was almost six times higher in this polluted area than the mean concentration of this element in samples collected in an unpolluted area. The levels of Mn in the unpolluted area were close to the regional background level of the element (Carballeira et al., 2002). It is therefore evident that the concentrations of Mn in moss samples from the surroundings of the aluminium smelter were much lower than usual for the region.

Furthermore, the results of the analysis of changes in the concentrations of Mn in the moss after transplanting samples from a contaminated to an unpolluted area showed an increase in the concentrations of Mn (up to 5 times in 2 months) in the moss samples transplanted to the unpolluted area. On the contrary, the concentrations of Mn in the samples of moss transplanted to the surroundings of the aluminium smelter decreased by half in only 30 days and to below the regional background level in just over 8 months. It appears that if the experiment had been continued for a sufficiently long period, the concentrations in samples transplanted within and between each of the sites would probably have equalized, so that the uptake of Mn cannot be due to genetic differences between the moss, but rather to differences in the environment where they are growing.

The low concentrations of Mn in the tissues of the moss samples growing in the surroundings of the aluminium smelter may be due to: i) lower inputs of Mn in the surroundings of the aluminium smelter than in the rest of the region, and ii) the existence of some process that prevents the moss capturing the Mn from the surroundings. With regard to the decrease in inputs, the main source of Mn for mosses is the surrounding vegetation. Some authors (Schaug et al., 1990; Berg et al., 1995a; Kuik and Wolterbeek, 1995; Steinnes, 1995; Fernández, 1999) who have used factorial analysis to compare levels of metals in mosses, found that one of the factorial axes only included Mn (vegetation factor). Other authors (Čeburnis and Steinnes, 2000; Aboal et al., 2004) found a significant correlation between the concentration of Mn in samples of moss and in conifer needles collected at the same SS,

and reached the conclusion that higher plants accumulate Mn through the root system, and that the element is then transferred to terrestrial mosses by leaching from both live and dead plant tissues. If the concentrations of Mn in the vegetation growing in the surroundings of an aluminium smelter are lower than in other areas, the inputs to moss will also be lower. However, there were no differences in the concentrations in trees growing in the surroundings of the smelter (147 $\mu g \cdot g^{-1}$ in needles of *Pinus pinaster* and 316 $\mu g \cdot g^{-1}$ in leaves of *Eucalyptus globulus*) and control trees (120 $\mu g \cdot g^{-1}$ in *P. pinaster* and 350 $\mu g \cdot g^{-1}$ in *E. globulus*) (E. Álvarez, pers. comm.), so that the first hypothesis can be ruled out.

In order to test the second hypothesis, the factor causing the reduction in uptake of Mn must be established. The high emission of F from the electrolytic cells in the smelter distinguishes the area surrounding the smelter from other areas. Experimental incubations were carried out to investigate the possible inhibition of uptake of Mn, caused by F (by a process of complexation). As incubation with F was carried out in a solution of FNa, an attempt was also made to distinguish the effect of F from that produced by Na, by carrying out another incubation in Na_2SO_4, as it is known that Na is capable of displacing Mn from the cation exchange sites in moss tissues (Gjengedal and Steinnes 1990; Schaug et al., 1990; Steinnes, 1995). The results did not show any evidence of inhibition of uptake of Mn, i.e. F did not appear to prevent uptake of Mn. As this experiment was limited to studying the effect of F, we decided to test the effect of samples of bulk precipitation originating from the surroundings of the aluminium smelter, where there is inhibition of the uptake of Mn, and in an unpolluted area where there is no such inhibition. In accordance with the results, which do not show any differences between both samples of bulk precipitation, it can be concluded that the experimental design of the incubations did not adequately reproduce the real environmental conditions under which inhibition of Mn uptake occurs.

Independently of the reasons for the inhibition of Mn uptake, it is clear that the linearity of the relationship between exposure and concentration

is altered by some as yet unknown reason (i.e. physicochemical characteristics of the emissions, physicochemical and biological processes and their interactions with environmental factors). It is therefore possible to explain the significance of the lack of correlation between the concentrations of Mn in atmospheric deposition and in the moss reported in the relevant literature. There are various possible causes of this lack of correlation: i) Mn is an essential element for moss and therefore moss has a high capacity to regulate the levels of this element, unlike those of other contaminants, although at high concentrations Mn may cause toxic effects (El-Jaoual and Cox, 1998); ii) there is evidence that the main source of Mn for moss is the surrounding vegetation, and not the atmosphere. Furthermore, according to Okland et al., (1999) in periods of water saturation, the soil surface can be another source of Mn for terrestrial mosses; iii) the chemical composition of the bulk precipitation may modify the concentrations of Mn in the moss tissue, as suggested in the relevant literature, although we were not able to determine this in the present study. Manganese is readily displaced from cation exchange sites in moss tissue, by other cations such as e.g. cations originating from sea spray (mainly Na^{+} and Mg^{+2}) and present in precipitation (Gjengedal and Steinnes, 1990; Schaug et al., 1990; Steinnes, 1995; De Caritat et al., 2001), and may also be complexed. This is not the explanation for the pattern of Mn observed in the surroundings of the aluminium smelter because: i) the concentrations of Na and Mg in moss in the studied area are not related to the distance to the sea; and ii) there is not any significant relationship between Mn and Na or Mg concentrations in mosses. Taking into account this result, we can conclude that Mn concentrations in mosses are not due to the displacement of cations exchanged produced by a Na or Mg gradient related to the distance to sea as the authors mentioned above.
The results obtained showed that on only two of the 50 occasions in which the method of detecting small scale emission points (section 3.5) was used, sources of emission of Mn were identified, unlike for the other metals and metalloids studied. There are two possible causes of this: i) that Mn is not usually emitted to the atmosphere from industries, and/or ii) although Mn

is emitted, there is no linear relationship between exposure and concentration, for the previously mentioned reasons. Therefore, we have to take into account that when we are talking about essential elements- the case of Mn- for plants, the concentrations of these elements that anthropogenic sources can emit to the atmosphere are not high enough to cause significant variations in the concentrations of these elements (i.e. Mn) in moss tissues (Reimann et al., 2006). Finally although Mn reaches the moss, it might not be taken up by the moss. After its extraction from pyrolusite (MnO_2), use of Mn is restricted to very few industrial processes, such as primary production of steel or the production of fertilizers and paints (Vukojevic et al., 2009), so that there are very few sources of emission of Mn to the atmosphere. The factory identified as a source of Mn is ferro-alloy factory, located in the west of the study region, and with a capacity to produce 50,000 $t \cdot year^{-1}$ of ferromanganese and 30,000 $t \cdot year^{-1}$ of silicomanganese. In this case, the moss would have functioned as a biomonitor of atmospheric Mn, in addition to displaying extremely high concentrations of Mn in its tissues. However, the nature of the contamination is mainly particulate, so that some of the processes already mentioned as preventing terrestrial mosses from being good biomonitors of atmospheric Mn will not be applicable. In this case only the capacity to retain particulate material will be important, and the biological and chemical processes that may affect the surface of the moss will be less so. Although terrestrial moss is not ideal as a biomonitor of atmospheric inputs of Mn, because it produces false positive results, it may be used under certain circumstances (i.e. high quantity of particulate material).

5. Conclusions

On the basis of the results obtained, we consider that biomonitoring with terrestrial mosses is not a reliable tool for the analysis of atmospheric deposition of Mn. Only exceptionally, under certain circumstances and for certain types of emission, increases in concentrations of Mn will be observed in moss tissues, in relation to increased deposition of the element. We therefore recommend the exclusion of this element from regional surveys, as also

proposed by other authors (Ross, 1990; Ceburnis et al., 1999).

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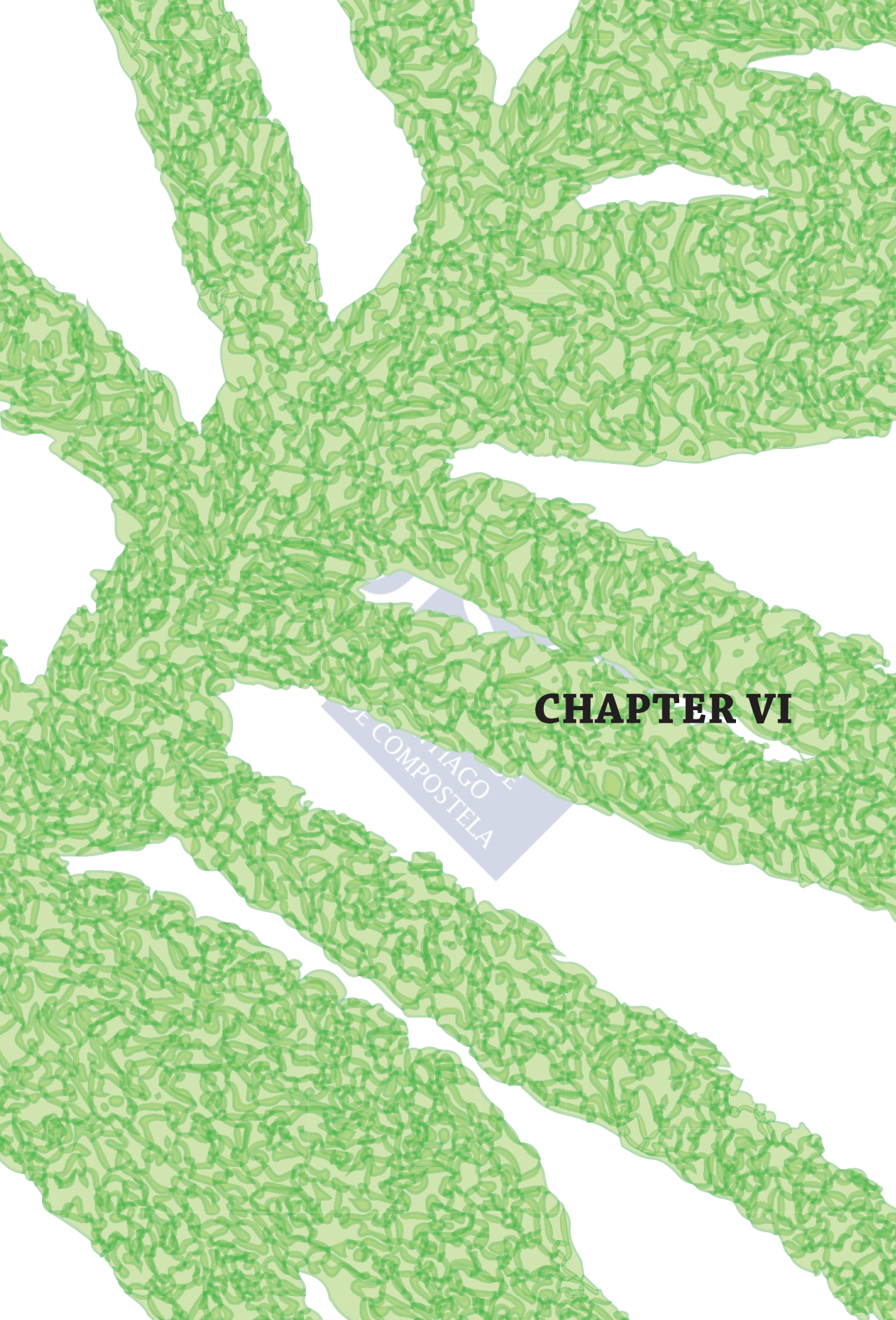
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CHAPTER VI

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Relationship between trace element concentrations in the terrestrial moss *Pseudoscleropodium purum* and in bulk deposition

Submitted for publication

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Abstract

We examined the relationship between the concentrations of Cd, Cu, Hg, Pb and Zn in the moss *Pseudoscleropodium purum* (Hedw.) M.M. Fleish and in bulk deposition (BD) at 21 sites (SS) affected by different levels of pollution (monthly sampling, January - December 2010). The aim of the study was to ascertain whether the lack of correlations reported in previous studies are due to limitations in the experimental design. We implemented some improvements to the experimental design and examined the above-mentioned correlations in greater depth. We found that i) the number of significant correlations was very limited and varied depending on how the data were analysed; ii) the regressions (slope and sign) varied when the combinations of moss and BD replicates used for comparison were changed randomly, even though the replicate samples of both materials were collected within a small area; iii) maximum bioconcentration factors (maximum concentrations reached in moss for a minimum level in BD) varied widely between elements, SS and time of exposure; and iv) the correlations between the concentrations of some elements (Cd and Cu, but not Hg, Pb or Zn) in moss tissue and BD increased with the number of months of accumulated BD data included in the analysis. We therefore deduce that the relationship is mainly determined by factors other than the inputs of the pollutants themselves. We conclude that native terrestrial mosses are less than ideal biomonitors for obtaining absolute values of atmospheric heavy metal deposition, and we recommend their use as qualitative indicators in biomonitoring studies and the use of classical methods when absolute values are needed.

Keywords: *biomonitoring, air quality, heavy metals, correlations.*

1. Introduction

Air pollution has been of great concern since the 1970s because of its possible harmful effects on both human health and natural ecosystems. The level of concern is illustrated by the number of laws created with the aim of preventing and controlling air pollution (e.g. Directives 2004/107/EC – relating to As, Cd, Hg, Ni and polycyclic aromatic hydrocarbons in ambient air – and 2008/50/EC – on ambient air quality and cleaner air for Europe – in the European Union, and the Clean Air Act in the United States, etc.). One of the most important aspects of the assessment of air quality is the establishment of accurate standardized measurement techniques that provide comparable information (Directive 2004/107/EC, also called the Fourth Daughter Directive). Reference methods for measuring atmospheric deposition of trace elements are usually based on classical measurements (i.e. chemical analysis of precipitation, particles and gases), which require the use of very expensive equipment and are sometimes not sensitive enough to detect the very low concentrations of these elements in the air (Wolterbeek, 2002). Such problems can be overcome by using biomonitors, i.e. organisms that provide quantitative information about the environment in which they grow (Markert et al., 1999). In fact, some regulations even consider the use of these kinds of organisms for assessment of regional patterns of impacts on ecosystems (Fourth Daughter Directive).

Terrestrial mosses have been extensively used as biomonitors for assessing air quality as they are geographically widespread species with no or poorly developed cuticle, no root system, and a high cation exchange capacity (Szczepaniak and Biziuk, 2003). Mosses also display a high capacity to retain particles in their tissues. Because of these characteristics, the element concentrations in mosses are at least partly related to their concentrations in atmospheric inputs. In the most recent ICP Vegetation Annual Report 2012/2013, the moss biomonitoring technique has been described as a complementary measure of heavy metal deposition from the atmosphere to terrestrial systems (which is easier and cheaper to apply than conventional pre-

cipitation analysis, thus enabling a high sampling density) (Harmens et al., 2013). Some researchers have attempted to calibrate moss concentrations against bulk deposition to obtain absolute deposition values (see e.g. Ross, 1990; Berg et al., 1995; Berg and Steinnes, 1997). Such calibrations would indicate whether significant correlations between both variables exist, the strength of the relationship (the percentage of the variability in moss concentrations explained by the concentrations in bulk deposition) and, thus, the degree to which element concentrations in mosses depend on the level of these elements in the bulk deposition rather than other processes. However, the results of these studies have revealed a low number of significant correlations. Moreover, the coefficient of determination usually explains a low proportion of the variability in moss data (Aboal et al., 2010).

Nevertheless, it is not clear whether these results are due to a weak relationship between element concentrations in moss and bulk deposition or to some methodological problems that limit the conclusions that can be reached. Aboal et al (2010) have already highlighted the following problems: i) use of a small number of sampling sites (SS) for the regressions (never more than 13); ii) use of a very limited range of concentrations (experiments almost exclusively carried out in unpolluted or slightly polluted SS); iii) deletion of some regression data assumed to be outliers; iv) use of moss and bulk deposition samples collected 1 km apart (or even more) from each other; v) use of more than one moss species in the same study; and vi) comparison of mean annual concentrations of bulk deposition and concentrations in moss samples collected after the bulk deposition sampling.

The aim of this study was to explore the relationship between the concentrations of some trace elements in mosses and in bulk deposition in greater detail than before. A series of improvements to the experimental design were implemented with the aim of clarifying the relationship between the moss and bulk deposition data.

2. Material and Methods

The study was carried out in Galicia (NW Spain), at 21 sampling sites (SS)

affected by different levels of pollution (i.e. ranging from unpolluted sites to highly polluted industrial areas – see Fig. 1). More specifically, the SS included areas close to two steelworks (SS1 and SS8-SS11), one woodworks (SS2), two coal fired power plants (SS3-SS7), an aluminium smelter (SS12-SS14), three peri-urban (SS15-SS17) and four rural areas (SS18-SS21). Samples of bulk deposition and of the terrestrial moss *Pseudoscleropodium purum* (Hedw.) M. Fleisch were collected monthly throughout 1 year (January–December, 2010).

2.1 Collection of bulk deposition and moss samples.

Three bulk deposition collectors, consisting of a funnel-bottle combination, were placed in each SS. Both the funnel (22 cm diameter and covered with a 0.5 cm plastic mesh to prevent the entry of plant and animal remains) and the bottle were made of opaque metal-free materials, to prevent the growth of algae. Prior to exposure, all of the collectors were washed with HNO_3 (20%). Sampling was carried out in accordance with the recommendations outlined in the Ambient air quality - standard method for determination of arsenic, cadmium, lead and nickel in atmospheric deposition: UNE-EN-standard 15841:2010 established by the European Committee for Standardization (CEN).

After each exposure period, the funnel was rinsed (into the bottle) with 100 mL of bidistilled water, to remove any particles adhered to the surface. The contents of the bottle were shaken and the total volume of precipitation in each collector was recorded. Finally, an aliquot of bulk deposition was acidified with hyperpure HNO_3 (final concentration of 1%) and stored at – 20ºC until analysis.

In addition, three samples of moss were collected at each SS, as close as possible to the bulk-deposition samplers (the closest sample was obtained at a distance of between 0 and 312 m from the BD sampler). Moss samples were collected in open areas, and each sample comprised more than 30 subsamples of similar weight and distributed uniformly throughout areas of approximately 30×30 m (Fernández et al., 2002, Aboal et al., 2006, Varela

et al., 2010).

2.2. Processing and chemical analysis.

The concentrations of Cd, Cu, Hg, Pb and Zn in the moss and bulk deposition samples were determined. These elements were selected either because they are very toxic to living organisms (Cd, Hg, Pb) or because significant correlations between the concentration in mosses and bulk deposition have frequently been reported. Moreover, despite the environmental and public health concern about mercury pollution, this element has hardly ever been included in this type of study.

The concentrations of Cd, Cu, Pb and Zn in bulk deposition samples were determined by inductively coupled plasma mass spectrometry (ICP-MS – VARIAN 820-MS quadrupole spectrometer) (at the Research Support Services Unit, University of Santiago de Compostela). Mercury was determined (after filtration of the samples through a 45 μm filter) by ICP-MS (NexION® 300X, Perkin Elmer Inc., Shelton, CT) (in the Department of Analytical Chemistry, Nutrition and Bromatology, University of Santiago de Compostela). For control of analytical quality, reference materials (MR1000 and MR10000 for Cd, Cu, Pb, Zn and Environment Canada Total Mercury PT97-Hg07 for Hg) were analysed in parallel to the bulk deposition samples. Percentages of recovery ranged between 67% (Cu) and 108% (Cd) for Cd, Cu, Pb and Zn, and were around 103% for Hg. The concentrations of all elements were above the limits of quantification (LOQ), with the exception of Cd (in 9% of the cases) and Hg (in 56% of the cases).

The moss samples were cleaned prior to analysis by removing plant remains and other foreign material, and green parts were separated from the other parts of the moss shoots. The samples were then homogenised in an ultracentrifugal metal-free mill (Retsch ZM 100) and dried at 45ºC until constant weight. Heavy metals were extracted by acid treatment (2% HNO_3 v/v) and ultrasound (SONICS Vibra cell VCX 130). The concentrations of Cd, Cu, and Pb were determined by graphite furnace atomic absorption spectrometry (Perkin Elmer AAnalyst 600). The concentrations of Zn were deter-

mined by flame atomic absorption spectrometry (Perkin Elmer 2100), and the concentrations of Hg were determined in an elemental analyzer (Milestone DMA80). For analytical quality control, duplicate samples were analysed once every nine samples. Two certified reference materials, M2 and M3 (*Pleurozium schreberi*, Steinnes et al., 1997), and analytical blanks (to control for possible contamination) were analysed at the same frequency. The overall percentage error (Ceburnis and Steinnes, 2000) was lower than 9% for all elements except Cu (15%). The percentage of recovery ranged between 75% (Hg in M2) and 101% (Pb in M3) and in most cases was higher than 89%.

2.3. Data analysis.

The mean concentrations (n = 3 replicates, except for Hg in BD) of each element in the moss tissues and in bulk deposition were grouped both by SS (n = 12) and by sampling time (n = 21). The normality of the data grouped in both ways was checked by means of the Shapiro-Wilks test, revealing that the concentrations of elements in bulk deposition and mosses were not usually normally distributed (in 24 - 95% and 10 – 100% of cases respectively), even when data were transformed (ln x, 1/x, $1/\sqrt{x}$). Therefore, the non parametric Spearman's rank correlation test was used to assess the strength of the relationship between the mean concentrations of elements in mosses and bulk deposition in the data grouped by SS and time.

The 3 replicate values per element corresponding to bulk deposition and moss samples were used to randomize (n = 25) the pairwise combinations (1 replicate of bulk deposition and 1 replicate of moss data) corresponding to the SS and times that yielded significant results in the previous analysis.

As mentioned above, Spearman's correlation coefficients were obtained for the regressions of the concentrations of each element in moss tissues and the accumulated bulk deposition, for different periods of time. For this purpose, bulk deposition data were summed monthly, bimonthly, quarterly, etc. until the accumulation of the bulk deposition for the whole year was compared with the concentrations of elements in mosses collected in the final sampling period (December 2010).

All statistical analysis were carried out using Microsoft Excel 2007 and IBM SPSS Statistics 20 software.

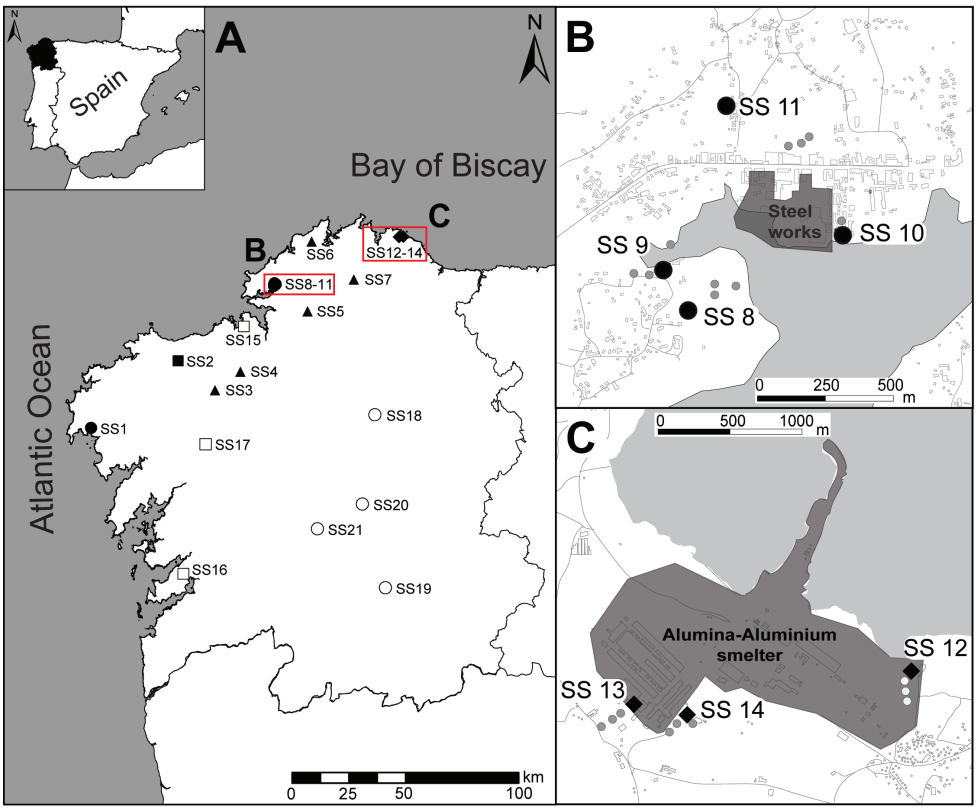


Figure 1: Map showing the location of the 21 sampling sites (SS) within the study region (A), and a detailed view of the various SS in the surroundings of a steelworks (B) and of the aluminium smelter (C). In the enlargements, the locations of the bulk deposition collectors are indicated by filled circles (B) and rhombii (C); the locations of the sites where native mosses were collected are indicated by grey circles. Filled circles: steelworks; filled square: woodworks; filled triangles: coal fired power plant; filled rhombus: aluminium smelter; empty squares: periurban areas; empty circles: unpolluted sites.

3. Results

The values of the Spearman's correlation coefficients and the degree of significance for the data grouped by SS and by sampling time are shown in Tables 1 and 2 respectively. For the data grouped by SS, very few of the correlations were significant, and half of these were negative. However, when the

concentrations of elements were grouped by time, the number of significant correlations increased drastically for Cd, Cu and Pb.

Figure 2 shows the graphs corresponding to some representative correla-

Table 1: Spearman's correlation coefficients for the concentrations of elements in bulk deposition and in moss tissues corresponding to the data grouped by sampling site (n = 12).
*: $p < 0.05$; **: $p < 0.01$.

SS	Cd	Cu	Hg	Pb	Zn
SS1	0.699*	-0.552	-0.400	0.259	-0.161
SS2	-0.406	-0.322	0.400	0.308	0.420
SS3	-0.321	-0.552	0.200	0.063	0.483
SS4	-0.413	-0.730	0.800	-0.301	0.287
SS5	-0.006	-0.329	-0.200	-0.021	-0.385
SS6	-0.006	-0.364	-0.771	0.371	-0.252
SS7	0.515	-0.301	0.900*	-0.196	-0.650*
SS8	-0.608*	0.049	-0.214	0.070	-0.699*
SS9	-0.566	0.014	-0.500	0.084	-0.049
SS10	-0.077	0.147	0.143	0.070	0.112
SS11	0.140	-0.301	0.543	0.259	0.287
SS12	0.188	-0.517	-0.393	0.217	-0.056
SS13	-0.392	0.035	-0.500	-0.035	0.182
SS14	0.168	-0.280	0.800	0.084	0.259
SS15	-0.245	0.021	-0.500	0.189	0.028
SS16	-0.009	-0.070	-0.800	0.154	-0.301
SS17	-0.145	0.147	-0.100	0.476	-0.343
SS18	0.333	0.287	0.900*	0.308	0.315
SS19	0.109	-0.077	0.800	0.161	0.266
SS20	-0.510	-0.210	0.000	-0.119	0.252
SS21	0.218	0.035	0.400	0.259	0.294

tions for each element, both for the data grouped by SS (Fig. 2A) and time (Fig. 2B). Each of these graphs also includes the line of maximum slope and its equation. This line was obtained by plotting the maximum concentrations of elements in moss, for a minimum value of bulk deposition, against the regional background level of each element (Carballeira et al., 2002) when its

Table 2: Spearman's correlation coefficients for the concentrations of elements in bulk deposition and in moss tissues corresponding to the data grouped by sampling period (n = 21). *: p < 0.05; **: p < 0.01; ***: p < 0.001.

Month	Cd	Cu	Hg	Pb	Zn
January	0.545*	0.534*	0.310	0.623**	-0.206
February	0.625**	0.488*	0.111	0.612**	0.334
March	0.370	0.105	-0.500	0.558**	-0.304
April	0.655**	0.710***	-	0.657**	0.199
May	0.539*	0.516*	0.467	0.062	0.039
June	0.618**	0.573**	-	0.673***	0.425
July	0.748***	0.718***	0.191	0.600**	0.275
August	0.604**	0.640**	0.447	0.656**	0.075
September	0.382	0.603**	-0.800	0.714***	0.110
October	0.713***	0.304	0.067	0.523*	0.312
November	0.179	0.448*	-	0.527*	0.226
December	0.610*	0.481*	0.018	0.703***	0.221

concentration in bulk deposition tends towards zero (the line is not shown for Hg because all the concentrations of Hg in SS7 and SS8 were below the regional background level for this element). The slope of this line represents the maximum bioconcentration factor of *P. purum* for each element at each SS and time, and was maximal at SS1 and time 8 (T8) for Cd, T5 for Cu, T1 and T8 for Pb, and at SS8 for Zn. Most of the points in the regression are much lower than the line of maximum slope.

Figure 3 shows the 25 regression lines obtained from the randomization process for one representative SS and time per element (Fig. 3A1 and 3B1). The Spearman's correlation coefficients corresponding to those data, for all SS and times that were significant in the previous correlation analysis (see Tables 1 and 2) are also shown (Fig. 3A2 and 3B2). The relationships changed from positive to negative values (Cd-T12 and Cu-T2 and T11 in Fig. 3B2), and the significance level (Fig. 3A2 and 3B2) also varied for the same dataset.

The Spearman's correlation coefficients corresponding to the regressions

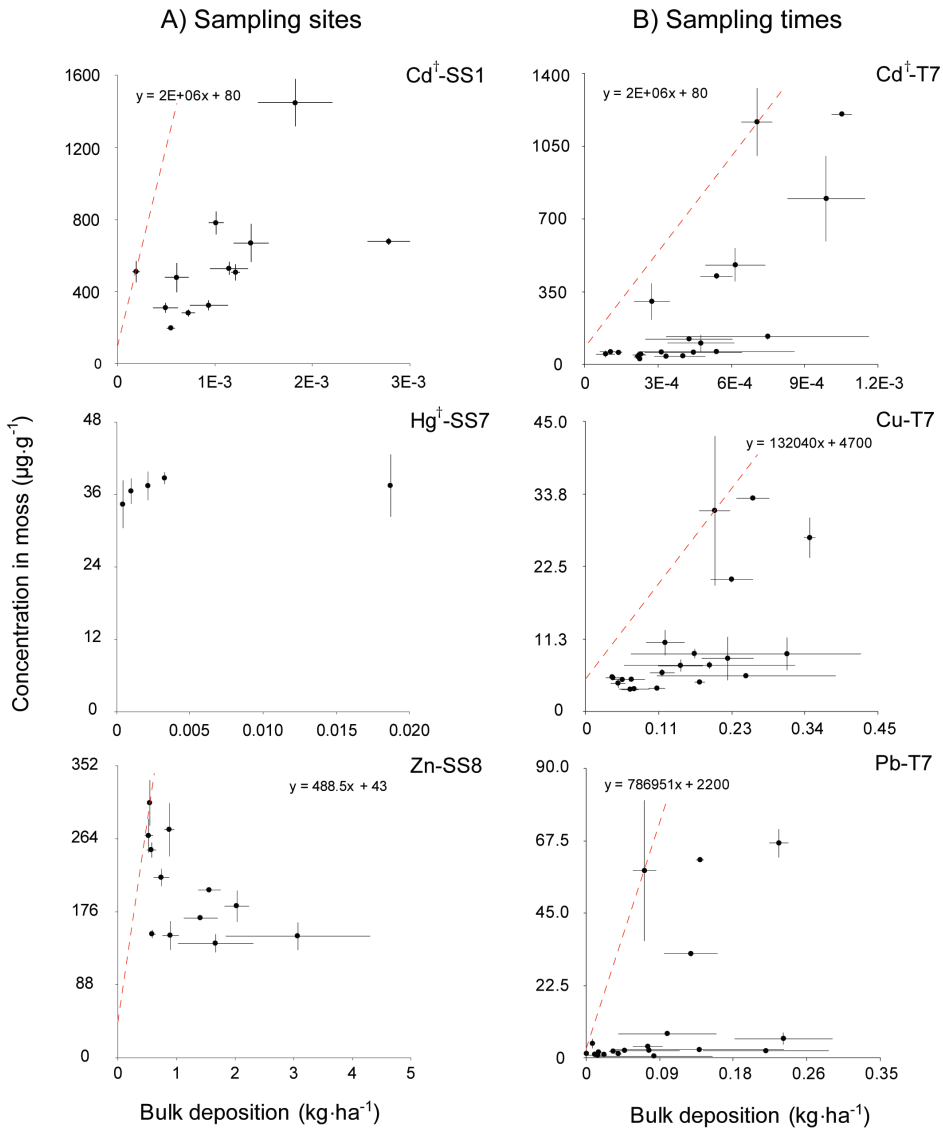


Figure 2: Graphs showing the concentrations of those elements for which significant correlations were observed between bulk deposition (BD; kg·ha^{-1}, g·ha^{-1} in the case of Hg) and moss tissues (µg·g^{-1}). The lines representing the maximum concentration in moss samples for a minimum level in BD, and the background levels in moss (when no elements are found in BD) for each element, together with their equations, are also shown (dashed red lines). The error bars correspond to the standard error calculated for n = 3 both in bulk deposition (horizontal bars) and in moss samples (vertical bars). †: concentrations in ng·g^{-1}. A) Data grouped by SS; B) Data grouped by time of collection).

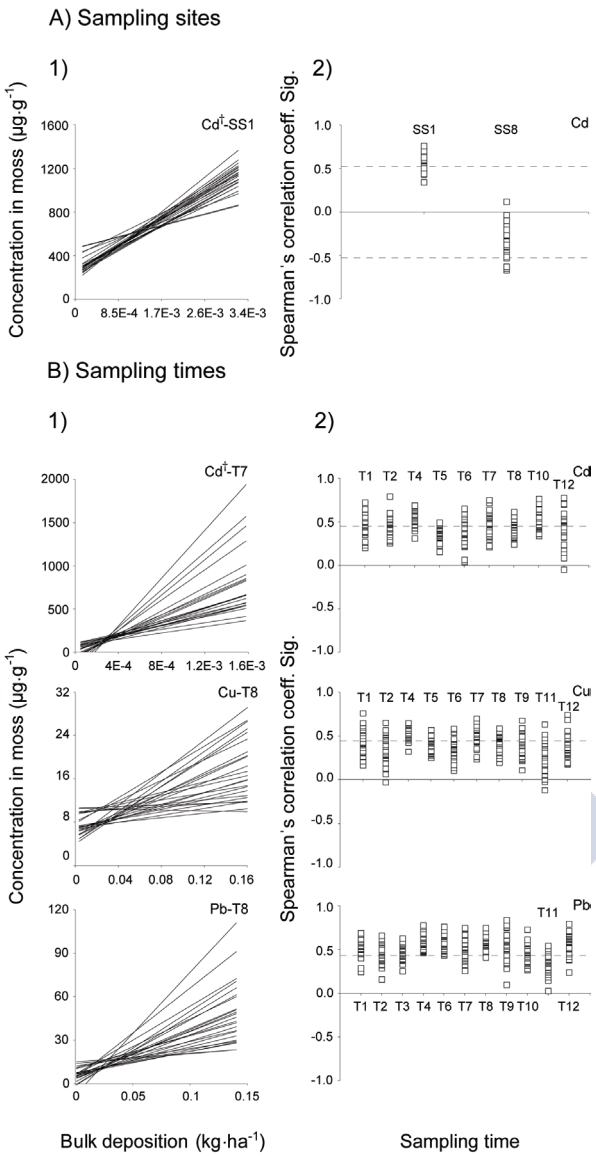


Figure 3: Graphs showing the 25 regression lines resulting from randomization of the combinations between the concentration of each element in moss (µg·g^{-1}) and bulk deposition (BD; kg·ha^{-1}) samples (n = 3 replicates), in one representative sampling site (SS) per element (1). Values of the Spearman's correlation coefficient (rho) obtained for each regression line, element and SS and time in which correlations between concentrations in moss and BD were significant are also shown (2). A) Data grouped by SS; B) Data grouped by time of collection). Dashed black lines: lowest value of rho that begins to be significant for $p < 0.05$. †: concentrations in ng·g^{-1}.

carried out with the bulk deposition accumulated over different periods of time (Fig. 4) clearly showed that the percentage of significant correlations increased with the number of months considered in the accumulated bulk deposition data on Cd and Cu (i.e. for Cd, the percentage increased from 58% for the monthly BD to 100% for the three-monthly BD). However, this pattern was less clear for Hg, Pb and Zn; for Hg, the percentage of significant correlations increased from 0 to 44% for regressions for BD accumulated over 4 months, but the inverse pattern was then observed (decrease with the increase in accumulated bulk deposition). For Pb, the decrease in the percentage of significant correlations with the increase of accumulated bulk deposition was clear from the onset (from 92% for monthly accumulated BD to 0% considering the BD accumulated over 8 months). Finally, no pattern was observed for Zn, as the percentage first decreased as the accumulated bulk deposition increased and then increased until the bulk deposition accumulated over 11 months.

4. Discussion

The use of moss to biomonitor air quality is based on the relationship between the concentrations of elements in moss and in bulk deposition. However, although the technique has been used for more than 50 years, this relationship has not been studied in depth (Aboal et al., 2010). In the few studies carried out with the aim of establishing the basis of this relationship, the results (i.e. the significance of the correlations between moss and bulk deposition data and the coefficients of determination) varied widely (Ross, 1990; Berg et al., 1995; Thöni et al., 1996; Berg and Steinnes, 1997; Schintu et al, 2005; Fowler et al., 2006). Moreover, several methodological limitations can be identified in most of these studies and therefore the conclusions should be considered with caution (Aboal et al., 2010). For all these reasons, we decided to implement several improvements to the experimental design with the aim of clarifying the characteristics of the relationship between the concentrations of some elements in moss and bulk deposition. The methodological aspects improved in this work include the following: i)

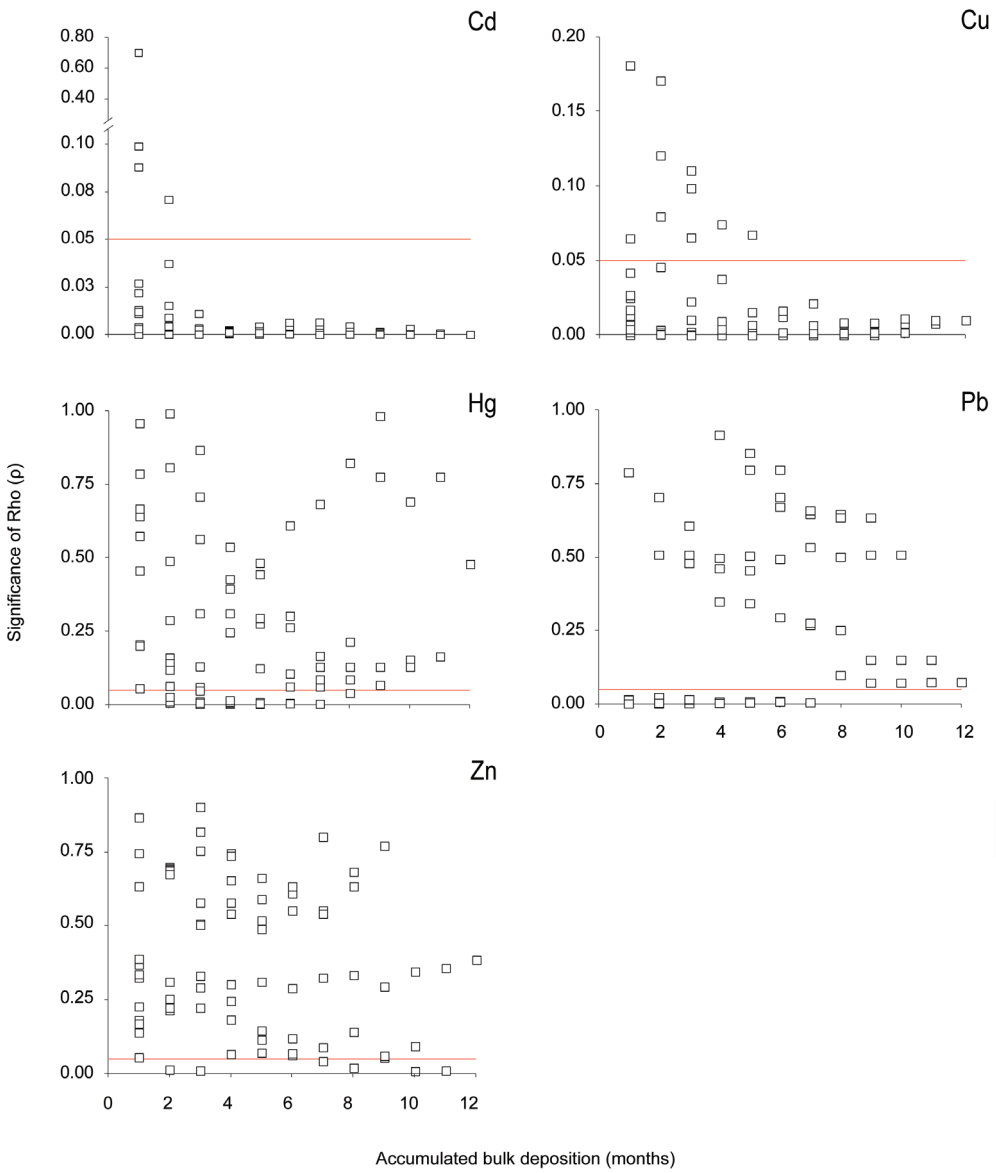


Figure 4: Degree of significance of the Spearman's correlation coefficient (rho) corresponding to the regressions of the concentrations of elements in moss tissues and the concentrations accumulated in the bulk deposition for different periods of time (n = 21). i.e. 1: regression analysis of the monthly concentrations; 2: regression analysis of the bimonthly accumulated concentrations; 3: regression analysis of the 3-month accumulated concentrations , etc. Solid red line: $p < 0.05$ level.

an increase in the number of SS to 21 SS, in comparison with the previous maximum of 13 SS (Berg et al., 1997); ii) use of sites affected by different

levels of pollution and inclusion of all the data in the analysis. In previous studies, unpolluted areas were included and highly polluted SS were excluded as they were considered outliers (i.e. Ross, 1990; Berg et al., 1995; Berg and Steinnes, 1997); iii) a reduction in the distance between the moss samples and the bulk deposition collectors. In this study, the maximum distance between the closest sample (of 3 moss samples collected per SS) and the collector was 312 m, and the maximum distance between any of the samples and the collector was 563 m. In previous studies, the distances ranged from 50-200 m (Thöni et al., 1996) up to 50 km (Berg et al., 1995); iv) moss sampling was synchronized with bulk deposition sampling, i.e. the moss samples were collected at the same time as bulk deposition (monthly). This enabled direct comparison of the concentrations of elements in both samples each month and also for longer periods, by summing the bulk deposition obtained for the whole study period (i.e. for between 2 and 12 months) and comparing it with the concentration of elements in the moss samples collected in the latest month. In previous studies, bulk deposition samples were collected every month and the mean annual concentrations were compared with element concentrations in moss samples collected at the end of the bulk deposition sampling period (i.e. Ross, 1990, Schintu et al., 2005). For example, bulk deposition collected during 1 year was compared with moss segments assumed to represent the tissues that had grown, and been exposed to atmospheric deposition, during the previous 3 years (i.e. Berg et al., 1995).

Despite the improvements made to the experimental design, the results of the present study appear to confirm that the relationship between the concentrations of elements in mosses and in bulk deposition is very complex and depends on factors other than the bulk deposition. In fact, very few significant correlations were found for the data grouped by SS (Table 1); when the data were grouped by sampling time (Table 2), the number of significant correlations increased greatly for Cd, Cu and Pb but was null for Hg and Zn. Grouping the data by SS should reduce the effect that the differences in the characteristics of the atmospheric inputs (e.g. particulate vs gaseous inputs,

particle size, etc.) will have on the final concentrations of elements in mosses, as all moss samples from the same site are exposed to the same sources of elements. However, this would increase the effect that differences in environmental factors (parameters such as temperature and amount of precipitation, which vary widely throughout the year) and the uptake capacity of the mosses (which depends on the physiological status of the organisms, which in turn varies with the environmental conditions) have on the final concentrations of elements in mosses (Boquete et al., 2014). Furthermore, the composition of the soluble and particulate fraction of bulk deposition changes throughout the year depending on the environmental conditions (Theodosi et al., 2010), which introduces yet another source of variability in the data.

On the contrary, grouping the data by time of exposure would homogenize the physiological status (according to the time of the year) and the general climatic conditions of the region. Therefore, the increase in the number of correlations when grouping the data by time of exposure would be explained by the following: i) differences in the effect of environmental factors on atmospheric inputs (particle solubilization or washout by precipitation events); ii) differences in the physiological status of moss (i.e. degree of hydration/dehydration, moss growth, etc.); and, iii) differences in capacity to take up elements (passive versus active uptake processes, cation exchange capacity, moss surface to area ratio, etc.). These differences alter the relationship between the concentrations in mosses and in bulk deposition to a greater extent than the differences in the way that elements reach these organisms (composition of atmospheric inputs, physicochemical interactions between elements and between elements and mosses, etc.). Nevertheless, the effects of all of the above-mentioned factors will depend on the study area (SS), time of the year (sampling time) and element considered, which would explain the lack of any consistent pattern of significant correlations between the concentrations of elements in moss and in bulk deposition regardless of the way in which the data were grouped.

The regression analyses conducted after randomization of the pairwise com-

binations (bulk deposition and moss data, as explained in the Material and Methods section) yielded a surface of 25 regression lines that varied in their slope and changed from positive to negative relationships. The correlation coefficients varied between non significant and strongly significant (note that these regression models could not be fitted to the data because of the lack of normality, but they are comparable to those presented in the available literature) (Fig. 3). The maximum distance between moss replicates was 480 m (but less than 50 m in 53% of the cases). Therefore, all replicates were close enough together to be exposed to the same sources of bulk deposition. However, according to Zechmeister (1995), moss physiology depends to a great extent on small scale specific climatic variables (i.e. microsite temperature, humidity, solar radiation, etc.). Among other parameters, this would determine moss growth, which would also determine the time that moss tissues had been exposed to atmospheric deposition of elements, and the proportion old/new tissues would determine the heavy metal uptake capacity of mosses (Boquete et al., 2014).

The differences in the relationships between the concentrations of elements in moss and in bulk deposition when different combinations of moss and bulk deposition were compared appear to be due to the microclimate effects on the uptake/retention capacity of mosses, or even the pool of elements that they receive at each specific location (within the same SS), as shown in the former paragraph. Thus, the implications of this are that in a normal biomonitoring study with terrestrial moss, in which just one moss sample is collected per SS, the degree to which this sample reflects atmospheric heavy metal deposition will depend on the exact location of the sample (even when restricted to a small area i.e. 1 km^2). Thus, the quality of the results obtained will vary randomly.

Comparison of the concentrations of trace elements in moss and bulk deposition enabled calculation of the maximum bioconcentration factors (some examples are shown in Fig. 2). These varied between elements (i.e. Cd>Pb>Cu>Zn), between SS (i.e. from 84.7 in SS7 to 488.5 in SS8 for Zn) and between times (e.g. from 1,000,000 in T5 to 5,000,000 in T8 for Cd). Dif-

ferences in the maximum bioconcentration factors for different elements may be explained by the variation in uptake and retention efficiencies of the different elements within the same moss species depending on their physicochemical characteristics. Thus, the high bioconcentration factors for Cd, Cu, Hg and Pb were probably due to the high covalent indexes of these elements (Nieboer and Richardson, 1980). This would help the elements to form strong bonds with moss cell walls and the outer layer of the plasma membrane, so that they will not be easily displaced by other elements. However, Zn (for which regional background levels were highest) is an essential nutrient and its uptake will greatly depend on the physiological needs of mosses. This, together with the fact that the covalent index is lower than for the other elements, explains the minimal bioconcentration factor.

On the other hand, the levels of elements in mosses will also depend on the physicochemical characteristics of these organisms. Thus, González and Pokrovsky (2014) showed that binding sites in mosses can be assigned to carboxyl, phosphodiester, phosphoryl, amines and sulfydryl groups. The first three groups contain oxygen and therefore preferably bind elements with low covalence index (i.e. Zn). However, amines and sulfydryl groups, which do not contain oxygen, preferably bind elements with high covalence indexes (i.e. Cd, Hg, Pb, Cu). Thus, the chemical composition of moss structures will also generate differences in the bioconcentration factors for different elements. Finally, the concentrations of elements in bulk deposition samples will also depend on the solubility of the element. More soluble elements will be more easily retained at cation exchange sites, but will also be easily leached by precipitation (cation competition, anion complexation, etc.). The above-mentioned differences will also depend on the balance between these processes.

The variations in the maximum bioconcentration factor between SS may be due, above all, to differences in the elements that reach the moss, that is, atmospheric inputs. Thus, according to Boquete et al. (2011), the form in which a contaminant is emitted (e.g. gaseous, bound to particles, etc.) and the presence of other elements in the bulk deposition will determine pro-

cesses such as solubilization and leaching of elements, cation competition, anionic complexation etc. which, in turn, will influence the preferential accumulation of some elements. The rate and type of moss growth, its physiological activity, phenotypic adaptations, etc., will vary at each SS and will also have an effect on moss uptake/retention capacity. It is known that the accumulation capacity of mosses differs depending on the range of concentrations of the pollutants in the environment (i.e. due to saturation effects such as those reported by Galsomiès et al., 1999, 2003). This may explain the variability between SS in the maximum bioconcentration factors observed in the present study. Finally, all of the above-mentioned factors will vary depending on environmental conditions, which also explains the variability in the maximum bioconcentration factors between times of exposure for the same SS.

The fact that bioconcentration factors vary as explained above implies that the rate of accumulation of one element in the same moss species cannot be predicted or extrapolated between SS or between times, so that the regression between moss and bulk deposition data also cannot be extrapolated. This also explains why the rate of accumulation of elements in mosses reaches a maximum, given a low value of these elements in bulk deposition, although this rate is not then maintained, even though the concentrations of elements in the environment (bulk deposition) keep increasing (note the cloud of points under the line of maximum slope in Fig. 2).

The highest values of the maximum bioconcentration factor obtained for the data grouped by SS, regardless of which correlations were significant, corresponded to the surroundings of industrial areas, i.e. the most heavily polluted SS (SS1, SS8, 9, 10 and 11 and SS12, 13 and 14). This pattern was maintained when grouping the data by sampling time (with few exceptions the highest values were always found in SS8, 9, 10 and 11). This suggests that mosses that have continuously been exposed to high levels of atmospheric deposition of heavy metals have a higher capacity for bioconcentration than mosses growing in cleaner environments. This is not consistent with findings of a previous study carried out by our research team, involving

a cross transplant experiment at SS11, in which it was found that, under the same atmospheric deposition conditions, mosses growing naturally at this SS accumulated less Cd, Cu, Hg, Pb and Zn than mosses that had been transplanted from a clean area (Boquete et al., 2013). It was assumed that the mosses native to the polluted area (SS11) had undergone an adaptive response to high levels of atmospheric heavy metal deposition. Therefore, the only explanation for the findings of the present study is that some information about the concentrations of elements in bulk deposition has been lost (even considering that the acidification of bulk deposition samples should solubilise most of the elements found in particles, at least for the legislated pollutants, i.e. Cd, Pb). This would make even more sense given that a large proportion of the elements that reach mosses in industrial areas are attached to particles (according to Spagnuolo et al. 2013, the quantity of particles in SS11 may be more than 2 times higher than in a clean area in the same region).

The highest values of the maximum bioconcentration factors obtained for the data grouped by SS occurred in the same or the following month(s) (one or two months later), in which maximum growth was observed (growth of mosses was measured monthly at each SS according to Boquete et al., 2014). This provides evidence that active uptake processes in the new and metabolically more active tissues imply a higher uptake capacity (as suggested by Fernández et al., 2010) of toxic elements such as Cd, Hg and Pb (as they are present in the environment) and of essential elements such as Cu and Zn (as they are required to fulfil the essential needs of moss metabolism).

Finally, in this study, only green parts of *P. purum* were analysed, with the aim of reducing the effect of tissue ageing on the cation uptake/retention capacity (Boquete et al., 2014). The time that these parts of moss tissues are exposed to deposition of atmospheric trace elements may differ greatly between SS (Boquete et al., 2014). Indeed, in the aforementioned study, the length of *P. purum* shoots that were exposed to atmospheric deposition during one year (March 2008 – March 2009) varied between 2.9 and 7.9 cm in SS3, SS4, SS5, SS6, SS7, SS17 and SS21 (same SS as in the present study).

Therefore, it is clear is that at the beginning of this experiment (January 2010), most of the tissues analysed would have been exposed to atmospheric deposition of trace element during the previous year, and only a small portion of the green parts (between 0.12 and 0.58 cm of the *P. purum* shoot growth occurred during January 2010 in all SS) would have been exposed to the atmospheric inputs received in January 2010. However, at the end of the year a higher proportion of the tissues, between 1.3 and 4.9 cm (average annual growth for all the SS) of the green parts of moss shoots would have been exposed to the atmospheric inputs in 2010. This would explain the increase in the number of significant correlations between the concentrations in mosses and in bulk deposition with the increase in the number of months included in the accumulated bulk deposition for Cd and Cu. However, the different behaviour of Hg, Pb and Zn may indicate that, in general, the concentrations in mosses are not positively related to the time of exposure, as mosses do not integrate heavy metal inputs from atmospheric deposition (Boquete et al., 2014). This would at least partly explain previous reports of a lack of significant correlations between the data from parts of moss shoots supposed to represent the previous 2 or 3 years growth and the bulk deposition corresponding to one year (Ross, 1990; Berg et al., 1995).

5. Conclusions

The improvements made to the experimental design of this study, relative to the design of previous studies, served to minimise the variability in the results due to methodological aspects and thus to highlight the effect of all other factors. The results of the present study can therefore be considered much more reliable than previous findings.

The main results of the present study are summarized as follows: i) very few significant correlations for the data grouped by SS (n = 21 SS) and a considerable increase in the case of Cd, Cu and Pb for the data grouped by time of exposure (n = 12 months); ii) changes in the slope and sign of the relationship between moss and bulk deposition data when varying the combinations of moss and bulk deposition replicates compared; iii) large varia-

tions in the maximum bioconcentration factors for elements, SS and times of exposure; iv) no increase (for some elements) in the number of significant correlations between accumulated bulk deposition and moss data with the increase in the number of months of accumulated bulk deposition considered. It should be taken into account that, given the important role of the environmental factors in the concentrations of elements in moss tissues, these results could be slightly improved in arid regions where the effect of precipitation might not be as determinant as in the present region.

The results show that the relationship between trace element concentrations in moss and BD is determined by factors such as moss physiology, uptake capacity, adaptation to the environment, growth, chemical structure, pollutant physicochemical characteristics and environmental factors, including temperature, precipitation events and humidity, rather than by the pollutant inputs themselves (bulk deposition). The weight of each of these factors in the determination of this relationship might be different. Thus, it would be expected that the influence of environmental factors, mainly precipitation amount and composition, was greater than, for instance, the effect of cation competition or anion complexation (between heavy metals). Besides, the above mentioned factors vary both spatially and temporally in a way that cannot be predicted at a fine scale. Thus, whether or not the variables are significantly correlated will depend on the combination of these factors in a given area at a given time. It can be concluded that native terrestrial mosses are less than ideal biomonitors for yielding absolute values of atmospheric heavy metal deposition. Therefore, the moss biomonitoring technique is only recommended for qualitative biomonitoring of air quality, while classical methods are recommended when absolute values are needed.

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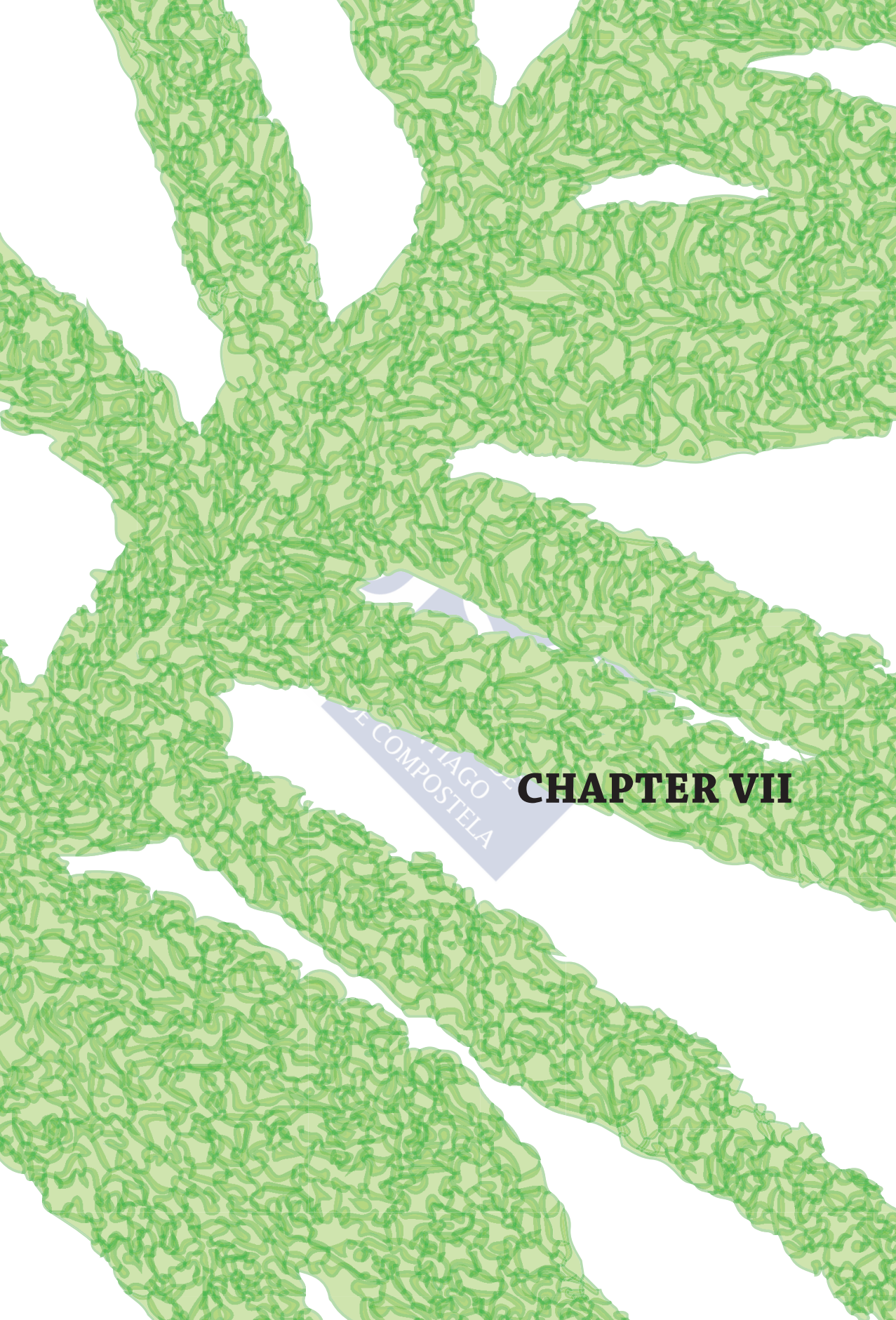
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CHAPTER VII

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A critical literature review on protocols for the moss biomonitoring technique

Submitted for publication.

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Abstract

Currently, the only existing guideline for the application of the moss technique to monitor the atmospheric deposition of heavy metals is the monitoring manual for heavy metals in European mosses published by the UNECE ICP Vegetation. Two main problems have been identified with this guideline: i) some of the recommendations regarding the methodological aspects involved in the application of the moss technique are not based on scientific criteria; and, ii) some recommendations in the manual are very vague and some aspects are even obviated. As a result there exist a high variability in the application of the protocol and many scientists adapt it to the specific conditions in the studied areas without evaluating how changes affect the results obtained. Therefore, in this review a total of 369 works were revised including both methodological and application studies of the passive biomonitoring of the atmospheric deposition of heavy metals with terrestrial mosses. The results have shown on the one hand, that none of the works completely accomplished the ICP-Vegetation protocol suggestions, either because the information regarding some aspects was lacking or simply because the authors did not follow the manual suggestions. On the other hand, it has been shown that the results of methodological studies sometimes contradict the ICP Vegetation manual recommendations. Thus, a new protocol in which each suggestion has been carefully and rigorously contrasted with the available literature has been proposed. Besides, practical and economical issues have also been considered and much more concise suggestions have been proposed which would facilitate its fulfilment in a more objective way.

Keywords: *passive biomonitoring, terrestrial mosses, heavy metals, methodology harmonization.*

1. Introduction

Passive biomonitoring with terrestrial mosses, i.e. the "moss biomonitoring technique", constitutes a useful tool for the study of air quality and,

more specifically, the atmospheric deposition of heavy metals. Despite the limitations already recognized in the scientific literature (e.g. Aboal et al., 2010), use of the technique enables researchers to distinguish (qualitatively or semiquantitatively) between contaminated and uncontaminated sites (Boquete et al., 2011a), to identify small scale pollution sources (Fernández et al., 2007), and to map spatial and temporal patterns of the heavy metals in the atmosphere (i.e. Bykowszczenko et al., 2006; Uyar, et al., 2007; Harmens et al., 2013). However, obtaining reliable, comparable and useful information requires a detailed, scientifically rigorous and homogenized protocol for the technique. All the steps involved in such a protocol, from the sampling design to sample processing and final storage, should be carefully described.

At present, the only existing guideline is the monitoring manual for heavy metals in European mosses, published by the UNECE ICP Vegetation (ICP-V). The most recent ICP monitoring manual, hereafter referred to as the ICP-V manual, was published for the 2015 European moss survey (Frontasyeva et al., 2014); however, the manual has remained substantially unchanged since its first version (Rühling, 1989). The manual provides a series of recommendations regarding the methodological aspects involved in the application of the moss biomonitoring technique. However, many of these recommendations are not based on scientific criteria, even though many studies have addressed methodological aspects of the technique. Thus, the effect of aspects such as the moss species used (e.g. Rinne and Makinen 1988; Wolterbeek et al., 1995; Ceburnis et al., 1997; Olajire 1998; Fernández et al., 2002a), the distance between sampling sites (SS) and vegetation (e.g. Steinnes 1995; Okland et al., 1999; Ceburnis and Steinnes 2000; Schröder et al., 2008) and the distance between SS and the pollution source (e.g. Mendil et al., 2005; Bignal et al., 2007; Carvajal et al., 2010) have been assessed in the final results obtained in biomonitoring studies. However, the outcomes of these studies are not always taken into account when elaborating the ICP-V manuals, as will be demonstrated throughout this review.

Some of the recommendations provided in the manual are very vague (e.g.

selection of the part of the moss shoot for analysis or the sampling period), and some aspects are even totally overlooked (i.e. the elevation and distance from the coast at which moss samples are collected). As a result, the protocol varies widely and many scientists adapt it to the specific conditions of a study area without taking into account the uncertainty that this may introduce in the final results of passive biomonitoring studies. Furthermore, many studies do not include information about how the technique was applied. For all these reasons it is very difficult to make valid comparisons of the results obtained in different studies. The conclusions that can be reached from such works remain rather limited even after more than 40 years of application of the technique. This is probably one of the main reasons why the moss technique is often viewed as an unreliable tool for environmental management and is not considered in environmental policy making.

The aim of this review is to propose a harmonized protocol for the application of the passive biomonitoring of air quality with terrestrial mosses. For this, a total of 461 papers published within the last 41 years (1972 - 2014) (identified by means of the Elsevier Scopus website) were taken into account. Of these, we did not have access to the full texts of 35 of the papers, 16 of which are published in non-indexed journals. After revision of the other 426 papers, 57 were disregarded because they did not focus on the use of terrestrial mosses for biomonitoring the atmospheric deposition of heavy metals. Thus, the final revision is based on the data extracted from a total of 369 articles. The following aspects involved in the application of the moss technique were studied in depth: i) pre-sampling issues, including number of sampling sites (SS), sampling design, survey frequency and moss species; ii) field sampling, including distance between the SS and vegetation, distance between the SS and possible sources of pollution, elevation, distance between the SS and the coast, representativeness of the SS, area occupied by the SS, number of subsamples, size (amount) of sample collected, sampling precautions and sampling period; iii) cleaning and storage, including sample treatment, selection of material, sample cleaning, sample drying, sample homogenization and final storage. We will discuss the effects

of each of these aspects on the final results of biomonitoring studies, the recommendations indicated in the ICP-V manual (Frontasyeva et al., 2014) and methodological studies focusing on each aspect, and we will propose a new, harmonized protocol.

Other additional and important aspects that affect the data have also been identified: chemical analysis of the samples and correction of moss concentrations considering soil concentrations. However, as these aspects are not directly involved in sample collection and preparation, which is the focus of this review, they will not be discussed here.

2. Pre-Sampling issues

2.1. Number of sampling sites

The number of sampling sites (SS) and their spatial arrangement (see section 2.2) are some of the most important aspects of the design of a biomonitoring study (Fernández et al., 2005; Cenci, 2008). The accuracy of the characterization of the pollution processes in a study area obviously increases with the number of SS considered; however, this considerably increases the cost of the method in terms of both time and money (Amblard-Gross et al., 2004). Therefore, the optimal number of SS should be defined on the basis of the degree of spatial resolution desired, on the scope of the work and on operational and economical constraints. Biomonitoring studies can generally be classified into two main groups: i) those focused on characterization of the pollution derived from focal points of emission (e.g. industrial facilities, mining areas, etc.); and ii) those focused on characterization of the general patterns of pollution in a wide area (i.e. at regional, national or transnational levels).

When the biomonitoring study is focused on the assessment of the pollution processes generated at a focal point, it is recommended that moss samples are collected around the facility, and that the number of SS should be decreased as the distance from the source increases (e.g. following a logarithmic grid; Bargagli, 1998). The number of SS will depend on the size of the area affected by the pollution process. According to Aboal et al. (2006a),

the area affected ranges in size between 6 and 40 km diameter for small and medium sized industries, and therefore the number of SS required will also be very variable. The lack of recommendations about the number of SS that should be sampled around this type of pollution source has resulted in a high degree of variability between different studies (Fig. 1A1).

In the case of extensive surveys, the ICP-V manual suggests a density of at least 1.5 moss samples/1000 km^2 for regional/national surveys. Alternatively, collection of 2 samples per EMEP grid (50 x 50 km) is also recommended (corresponding to approx. 0.8 sites/1000 km^2). Nevertheless, despite these recommendations, the sampling densities used in extensive surveys carried out between 1977 and 2010 were also very variable (Fig. 1A2). From a total of 201 articles revised, 13% included data from national surveys carried out in different countries, 16% reanalyzed previous data obtained in one or various national surveys, 11% focused on heavy metal deposition in some countries, and 60% corresponded to regional surveys (Fig. 1A3). Most of the studies complied with the ICP-V manual recommendations, while more than a third used much higher densities. However, the ICP-V manual does suggest a method of calculating the minimum sample size taking into account data from previous surveys.

None of the papers reviewed specifically considered the density of sampling sites that is most suitable for regional, national and transnational scale studies. However, some researchers investigated the most appropriate grid size for sampling networks (Cenci, 1998; Amblard-Gross et al., 2004; Fernández et al., 2005; Boquete et al., 2009), which can be used to estimate the optimal density of sampling sites. Cenci (1998) recommended the use of different grid sizes depending on the study objectives (i.e. 20 x 20 or 30 x 30 km, equivalent to approx. 2.5 and 1.1 sites/1000 km^2 respectively), without providing any justification for these options. Amblard-Gross et al. (2004) suggest the use of 30 x 30 km grid based on the variance of the data and the results of a cost-benefit analysis carried out using *Pseudoscleropodium purum*, *Pleurozium schreberi* and *Eurynchium praelongum*. According to these authors, sampling two points in squares of this size reduces the total vari-

ability in the concentrations of elements such as Pb and Sb, although more samples are required for other elements (i.e. Cu, Sr, Rb and Zn). However, in reaching these conclusions, the authors did not take into account the variance due to the spatial variability in the concentrations of the metals in the moss samples. Likewise, Pesch et al. (2008) developed a method of reducing the number of SS required in a monitoring network in Germany (by using a combination of 16 x 16 km and 25 x 25 km sampling grids, equivalent to approx. 4 and 1.6 sites/1000 km^2 respectively), with the aim of reducing the costs without affecting the representativeness of the samples. The recommendation made by Fernández et al. (2005) for use of 30 x 30 or 32 x 32 km sampling grids (approx. 1 site/ 1000 km^2) was based on the fact that these sizes (or fractions of these) are commonly used in other studies.

The study carried out by Boquete et al. (2009) in Galicia (NW Spain) with the moss species *P. purum* is the only one that specifically considers the grid size (and indirectly the number of SS), by studying how it interacts with the scale of the contamination process. After combined analysis of the results corresponding to different grid sizes, these authors concluded that for adequate characterization of large scale contamination processes (e.g. Cu, Hg and Se), sampling grids of 15 x 15 km (equivalent to 4.5 sites/1000 km^2) are required, whereas small scale processes (e.g. Cd, Pb and V) can only be detected with 1 x 1 km grids (approx. 1000 sites/1000 km^2) (which is totally impractical in large areas). Taking into account that in each area the contamination processes associated with each element vary according to the scale, these authors conclude that it is impossible to establish a suitable grid size (and therefore the number of SS) for all contaminants and zones and that this should be studied for each region on the basis of the spatial structure of the concentrations of the elements in moss.

The results of biomonitoring studies will depend on the densities of SS and hence only studies using the same densities can be compared. Thus, the ICP-V manual recommendation of two different densities is not practical. Moreover, the method recommended for calculating the minimum sample size assumes normality and stationarity (lack of spatial structure) in the

dataset. Finally, as the optimal SS density is specific for each pollutant and region, we conclude that a density of 1 site/1000 km^2, corresponding to a grid of 32 x 32 km, is the minimum advisable density (intermediate between the densities proposed by the ICP-V manual). When more detailed spatial resolution is required, a nested design with increasing density of SS is recommended: 16 x 16, 8 x 8, 4 x 4 km, etc. (equivalent to approx. 4, 16 and 62 sites/1000 km^2, respectively). Thus, more intensive sampling would be appropriate when local pollution processes that affect small areas (originated by point sources of emission) coexist with the general pattern of pollutants distribution in extensive areas.

2.2. Sampling design

The sampling strategy (i.e. linear transect, grid, etc.) and design (i.e. regular, random, stratified, etc.) are often overlooked in biomonitoring studies (Bealey et al., 2008). However, both aspects are extremely important as they will determine the final outcome of this type of study, and therefore, the level at which the scope of the work is fulfilled. Indeed, the first step when planning the sampling strategy and design for a biomonitoring study should be proposal of the study objectives (Wolterbeek and Bode, 1995). Moreover, as Aboal et al. (2006a) pointed out, the spatial layout of the SS will determine the type of pollution processes (i.e. small or large scale) that will be reflected by the moss samples. As a consequence, the use of different sampling strategies will preclude comparison of the results obtained in different surveys.

It has traditionally been recommended that for extensive studies moss samples should be collected from points distributed evenly throughout the region, with a higher density of sampling in areas where pronounced contamination gradients are expected (ICP-V manual). Fig. 1B shows the percentage of studies reviewed that used different sampling designs for local and extensive areas. In the first case, the most commonly used design was an even distribution of SS in the study area (in almost 50% of cases) followed by transects originating from the source (around 25%); however, in 25% of the

papers no information about the sampling design was provided. In the case of extensive surveys, the most common sampling strategy was again for evenly distributed sites, mainly in regional and trans-national studies.

Only one study (Fernández et al., 2005) has investigated the effect of the use of different sampling designs on the characterization of patterns of contamination in extensive areas. These authors used *P. purum* collected under three different sampling strategies in the same region: i) at the nodes of a regular grid, ii) at points within grid cells, and iii) using evenly distributed points. The authors concluded that the use of regular grids enables a) characterization of large scale regional patterns of contamination, b) identification of the position of the main focal points of emission, c) estimation of the size of the most contaminated areas; and d) comparison of the results obtained in different regions. They also concluded that use of the sampling design recommended by the ICP-V manual would lead to overestimation of the regional levels of metal pollution.

Taking into account the advantages of the use of regular grids indicated by Fernández et al. (2005), we consider that this would be the best sampling design for characterizing the atmospheric deposition of pollutants in a region. Regular sampling appears to be more appropriate than random sampling as it ensures an even distribution of the SS and the maintenance of the SS densities throughout the entire region.

2.3. Timing of surveys

The aim of repeating the sampling, in both extensive studies and those carried out in the surroundings of point sources of contamination, is to evaluate the possible changes in concentrations of elements in moss over time (years). No studies involving point sources of contamination have repeated the sampling over a period of years. In extensive-type studies, such as those carried out periodically in the European network coordinated by ICP Vegetation, sampling is carried out every 5 years (ICP-V manual). However, selection of this time interval is not based on the results of any previous scientific studies. According to Olsson and Bignert (1997), the frequency

of the surveys will depend on the intra-annual variability in the concentrations (see section 3.10), the magnitude of the trend (the differences between years) and on the size of the temporal series. As the number of years between surveys decreases, it will become easier to differentiate the natural random variation from any new trends. Even when the differences between years are negligible, continuing with the surveys is recommended as an early warning system for detection of increased presence of contaminants.

2.4. Moss species

This is one of the most important factors influencing the concentrations of elements in moss tissues (e.g. Kleppin et al., 2008; Schröeder et al., 2008; Holy et al., 2009). Researchers setting up large-scale survey often face the problem that the location of the SS becomes subordinate to the presence/absence of the selected species (Wolterbeek and Bode, 1995). This problem can be overcome by using more than one moss species within the same survey; however, it is clear that the concentrations of elements may vary considerably between species (e.g. Folkeson, 1979; Carballeira et al., 2008), thus precluding comparison of the results obtained. According to the ICP-V manual, only pleurocarpous mosses should be sampled and, of these, *Hylocomium splendens* and *P. schreberi* should be favoured, followed by *Hypnum cupressiforme* and *P. purum*. The use of other species (e.g. *Abietinella abietina* var. *Abietina* in mountainous areas or *Barbula indica* in arid regions) must be preceded by a comparison and calibration of element uptake relative to that in the main preferred species. Around 50% of the studies carried out worldwide used just one biomonitoring species (Fig. 1C1). Although studies involving a large number of species (9, 10, 14 or even 23) are very scarce, at least 5 or more species were used in 14% of the studies reviewed. However, the number of different moss genera used increased to nearly 90 (synonyms were disregarded on the basis of the information available in the Tropicos database). Of these, *Hylocomium*, *Pleurozium* and *Hypnum* were very common, while 90% of the genera were used on fewer than 10 occasions (Fig. 1C2, which also shows the number of different species used from each gen-

era). In 74% of the studies in which a maximum of two species were used: *P. schreberi* and/or *H. splendens* and/or *H. cupressiforme*.

Different studies have compared the concentrations of elements in pairs of species collected within the same SS (Table 1, supplementary material). The number of species involved in pairwise comparisons is very limited relative to the total number of species used. In a total of 13 articles, only 7 different pairwise comparisons were made: *P. schreberi* vs. *H. splendens* / *Brachythecium rutabulum* / *H. cupressiforme* / *P. purum* / *Rhytidialdelphus squarrosus* , *H. splendens* vs. *Sphagnum* spp, and *H. cupressiforme* vs. *P. purum*. The first of these comparisons is the most frequent, probably because both species are, as previously explained, the most commonly used in air pollution biomonitoring.

According to Halleraker et al. (1998), the results of paired comparison can be directly combined if the concentrations are significantly correlated (as indicated by Pearson´s or Spearman´s correlation coefficients) and if the species ratio (concentrations in Sp1 divided by concentrations in Sp2) does not differ significantly from 1 in the SS in which the species were sampled (as indicated by a t-test comparing the measurement unit with the unit of the distribution of the ratios between the species compared). Carballeira et al. (2008) suggested that when the regression line slope and elevation do not differ significantly from the line of slope 1 (covariance analysis), the species could be combined. Interspecies calibration has been recommended for situations when the concentrations are significantly different, but correlated (Reimann et al., 2001). The use of type II (calculated as standardized major axis regression) rather than type I regressions (often reported in the literature) is recommended because field values are used and none of the variables are independent (Carballeira et al., 2008). Conversion of the concentrations found in one species to those in another species has been carried out by using the ratio of concentrations in the two species as a correction factor (i.e. Halleraker et al., 1998). However, this ratio is not reliable because it varies depending on where the samples have been collected. Most studies involving interspecies comparison only assess the correlations be-

tween the species, and therefore no conclusions about their combined use can be reached (Table 1, supplementary material).

The elements for which the previous requirements are fulfilled vary from one study to another (Table 1, supplementary material), even when the same species are compared. For instance, data on Pb and Zn concentrations in *P. schreberi* and *H. splendens* were combined, taking into account the former criteria, in Canada and Germany (Barclay-Estrup and Rinne, 1978 and Wolterbeek et al., 1995 respectively), and the same is true for Cu, Fe, Ni in Germany. However, according to Halleraker et al. (1998), *P. schreberi*/*H. splendens* ratios of Cu, Fe and Ni are significantly different from 1 in the central Barents region. These results may be explained by differences in the accumulation capacity of bryophytes due to the range of concentrations of the pollutants in the environment. In fact, some authors have observed that the ratios of concentrations of the same element between species can vary from greater than to lower than 1 depending on the SS (Reimann et al., 2001).

Interspecies calibration is only valid for the range of concentrations considered and under the same chemogeographical conditions to those in which the intercalibration studies have been carried out; in other words, intercalibrations cannot be extrapolated (Wolterbeek and Bode, 1995). Moreover, there is some degree of uncertainty associated with the coefficients of the regression (Wolterbeek et al., 1995), which should be added to the uncertainty of the whole dataset. Most authors have therefore reached the conclusion that the interspecies calibration should not be conducted as it introduces further variability to the data (Thöni et al., 1996; Halleraker et al., 1998; Carballeira et al., 2008). In this respect, Carballeira et al. (2008) suggest only carrying out calibrations when the values of the regression coefficients are very high (i.e. 0.99).

Thus, although recommended by the ICP-V manual, the scientific evidence suggests that interspecies calibration of the uptake of heavy metals should not be applied. The differences in the levels of heavy metals concentrated by different moss species would prevent valid comparison of the results ob-

tained with these studies. When the use of more than one species is mandatory, this would limit interpretation of the data to a qualitative estimate of the levels of pollutants. However, the results from studies carried out in the same region will be comparable over time, and temporal patterns from different areas will be also be comparable.

3. Field Sampling

3.1. Distance between SS and vegetation

The concentrations of atmospherically-derived chemical elements are altered on passing through plant cover (including epiphytes). The difference in concentrations will depend on the type of vegetation, the climatic conditions, the physicochemical characteristics of the elements and the amount and type of deposition (wet or dry). The concentrations are altered by the following processes (see e.g. Aboal, et al., 2002): i) the higher capture efficiency of the plant cover relative to that of surfaces without cover (due to the greater degree of roughness); ii) leaching of elements from the plant tissues, exudates and decomposition products, as well as the ion exchange reactions in the plant cover; and iii) the capacity of plants to incorporate elements from the total deposition (e.g. via association with weak organic acids). As a result of the combination of all of these processes, the concentrations of ions will differ from those in areas with no plant cover. As well as the inputs of elements, these processes are also important because i) they may vary the chemical forms of elements and their bioavailability (Markert et al., 1999), and ii) they will determine possible new cation equilibria in the moss tissues (e.g. an increase in pH will decrease the displacement of protons from exchange sites, thus leading to an increase in the concentrations of heavy metals). The plant cover also affects the intensity, distribution, amount and potential energy of the rain that passes through the cover, thereby affecting the uptake processes (Markert et al., 1999).

Hence, it seems obvious that collection of moss under plant cover is not appropriate for estimation of the atmospheric deposition. The ICP-V manual specifies that samples should be collected in sites "*[...] at least 3 m away from*

the nearest projected tree canopy: in gaps of forests (diameter >10 m) or plantations primarily (diameter >5 m), without pronounced influence from canopy drip from trees, preferably on the ground or on the surface of decaying stumps" and "*In habitats such as open heathland, grassland or peatland, sampling below a canopy of shrubs or large-leafed herbs should be avoided, as well as areas with running water on slopes*". Likewise, Bargagli (1998) recommends that whenever possible, the sampling points should be at least 5 m from the closest tree, and that in shrubland or grassland with high plants, the moss samples should not be collected beside these plants. The German sampling guideline establishes a critical distance between SS and small forests of 100 m (Pesch and Schröeder, 2006). However, despite the important influence of vascular plant cover on the concentrations of elements in moss tissues, around half of the papers reviewed do not provide any information about the distance from vegetation at which moss samples were collected (Fig. 1D). Some studies follow the ICP-V manual, and others provide qualitative (Fig. 1D1) or quantitative information (Fig. 1D2); the SS are usually located 3 or 5 metres from the vegetation to avoid the influence of the canopy drip.

The water intercepted by the canopy will gather and flow downwards through the tree tissues and reach the soil after falling from the leaves, twigs and branches (throughfall), or after flowing down the tree trunks (stemflow) (Rutter et al. 1971). Both throughfall and stemflow generate areas beneath the canopy forest (hotspots) in which the water and solute fluxes are greatly increased (Germer et al., 2012); this is particularly true for stemflow due to a funnelling effect (see e.g. Aboal et al., in press). Different researchers have investigated how stemflow affects the concentrations of elements in moss, by comparing moss samples taken from tree trunks (exposed to stemflow) with others growing on soil or outside forests (Table 2, supplementary material). In general, these studies clearly showed that moss is affected by stemflow. Various researchers have studied the effects of throughfall by comparing the uptake in moss in areas exposed to canopy drip and in open areas (Table 2, supplementary material), and usually did not find any differences in elements. These results are contradictory, especially those for Mn,

to which special attention has been given as an element for which vascular plants act as sources for moss (Boquete et al., 2011b). The lack of differences may be due to the application of ANOVA rather than other tests designed for paired samples, such as Wilcoxon's test. As shown on reanalysis of the data obtained by Fernández and Carballeira (2002), the latter type of test may be more sensitive for detecting differences.

The vegetation not only affects the concentrations of elements in moss directly via the stemflow and throughfall, as the amount of deposition that reaches a forest or adjacent zone is influenced by the surrounding cover due to i) the effect of the aerodynamic resistance of the cover (as a function of the height of the vegetation), and ii) the "shading effect" of any vegetation adjacent to the moss (Varela et al., 2010). The effect of the adjacent vegetation on the moss is reported in the literature; thus e.g. Pesch and Schröeder (2006) detected a weak statistical association between the bioaccumulation of mosses and the distance from trees. Employing other data sets and statistical techniques, Schröeder et al. (2008) and Kleppin et al. (2008) stated that the canopy drip generated spatial variability in the metal accumulation. However, Holy et al. (2009) used the same statistical techniques but reached the opposite conclusion in the French survey. According to Sucharová and Suchara (1998), the concentration of the mainly geogenous elements Al, As, Co, Cr, Fe, Hg, S and V in the mosses decreased significantly ($p < 0.01$) with the percentage of forest cover in a 5-km radius around the sampling plots, indicating effective filtering of dust particles by forests. Bargagli (1995) found that the concentrations of lithophile elements (Al, Ba, Cr, Fe and Mn) in *P. purum* were significantly lower in open areas close to Mediterranean maquis and woods than those from barren and cultivated areas. Likewise, Bargagli et al. (1995) found differences in lithophile elements (Al, Fe and Mg) in *H. cupressiforme*, the concentrations of which were higher in pastoral areas than in wooded areas.

According to all the information presented above, it can be concluded that the vegetation will have a double effect on the concentrations of metals in mosses, and it seems that the precautions proposed by the ICP-V manual are

not restrictive enough to prevent this. On the one hand, the direct effect of vegetation could be prevented by separating the samples from the area of influence of stemflow, which can be described by the following equation: y = 23.63 ln(x) - 31.53, where x = diameter at the tree base (cm) and, y = radius of stemflow infiltration from the centre of the stem (cm) (Tanaka, 2011). On the other hand, the shading effect could be avoided by maintaining a distance between moss and vegetation, as far as possible and advisable, that is twice the height of the surrounding vegetation (below an angle of 22.5º), as recommended by the World Meteorological Organization for positioning a pluviometer, for correct measurement of precipitation (WMO-No.8, 1983). This recommendation should be applied when possible due to the limitations presented in section 3.6.

3.2 Distance between SS and pollution focus

As indicated at the beginning of section 2.2, the distance between possible point sources of contamination and the moss sampling sites will depend on the study objectives. If the aim is to characterize the contamination in the surroundings of contamination points (e.g. industries, motorways, etc.), the samples should be obtained close to the contamination source and at increasing distances from this point (see e.g. Bargagli, 1998). Nevertheless, if the objective is to characterize patterns of contamination patterns over a wide area (e.g. region, country, etc.), local sources should be avoided as they may distort the patterns. Local sources include small and medium sized industries, small urban nuclei and roads and motorways. It has been suggested that other sources not previously considered, such as railways and airports, should also be taken into account (Carvajal et al., 2010). In addition, the German sampling guidelines establish a critical distance from high voltage power lines of 200 m (Pesch and Schröeder, 2006). These authors also concluded that in Germany, metal accumulation in mosses is sometimes correlated with the distance between the SS and industrial plants, human settlements, roads and motorways.

The ICP-V manual indicates that in extensive surveys, sampling should be

carried out at sites representative of non-urban areas, at a distance of least 300 m from main roads (i.e. motorways), urban and industrial nuclei, and at least 100 m from smaller roads and isolated houses. Other protocols, such as that used in Germany (Pesch and Schröeder, 2006) establish greater distances of up to 1000 m from industries and 300 m from any human settlements. Nevertheless, although the aim of these measures is to avoid point sources of pollution, sound scientific arguments for doing so have never been provided. Review of only those studies involving extensive surveys (Fig. 1E) shows that information about the distance between the sampling points and contamination points is only provided in 65% of cases (17% following European guidelines and 48% specifying the distances). In this latter group of studies, different types of contamination sources are considered (e.g. secondary and main roads, individual houses, populated areas, industrial settlements, motorways, etc.) (Fig. 1E1, 1E2): for secondary roads and isolated houses, the most commonly used distance is 100 m; for main roads, motorways and populated areas, the distance is 300 m; and finally for other possible sources (e.g. industrial settlements, mining areas, etc.), the range of distances is very variable.

To establish the optimal distance between contamination points and SS, the best option is to study how the pollutant disperses around, by means of gradient sampling. Very few traffic-related studies of heavy metals in native mosses have been based on sampling designs along a gradient (Santelman and Gorham, 1988; Naszradi et al., 2004; Zechmeister et al., 2005, 2006). These studies have identified dispersion patterns for different elements (e.g. As, Cr, Cu, Fe, Mo, Ni, Pb, Pd, Pt, Rh, V and Zn), mainly within 300 m from roads. These elements are mainly emitted as a result of erosion of brakes and tyres, loss of oil and exhaust emissions (Zechmeister et al., 2006; Napier et al., 2008). Only two studies have addressed pollution gradients in populated areas: one in a small Finnish town (Hermann and Hübner, 1984) and the other in a Norwegian city (Oslo) (Reimann et al., 2006). In both studies, Cu, Fe, Pb and Zn were found to be associated with various sources of emissions (mainly from traffic, heating systems and industries), and the latter study

also detected Au, Cd, Co, Mo, Ni and Pt (Reimann et al., 2006). The distance over which the elements were detected depended on the size of the town; for the small town, the pollutants were usually dispersed over a distance of 3 km, and for the city (Oslo) the distance was much greater, of between 20 and 40 km from the city centre. Large cities may generate regional patterns of pollution and hence cannot be considered as local sources. The extent of the influence of different types of industries on the concentrations of metals in mosses growing in the surrounding areas has also been studied using gradient designs (see e.g. Türkan et al., 1995; Brumelis et al., 1999; Real et al., 2003; Fernández et al., 2004; Samecka-Cymerman et al., 2009; González-Miqueo et al., 2010), which give a precise indication of the distances over which the contaminants spread. In general, the available data show that the contaminants emitted from these sources can reach distances up to 4 km.

The effect of local pollution sources on the general spatial patterns of pollutant concentrations in more extensive areas has also been studied. Pesch and Schröder (2006) could not verify the effect of the distance from local sources on metal accumulation for all the studied elements in the German survey. However, the concentrations of some of the elements were significantly correlated with the distance from roads, motorways, human settlements and industrial plants. The authors pointed out that the regional deposition patterns may have a greater impact on metal accumulation than nearby emission sources, or that the class boundaries used to perform the analyses might not be appropriate. However, Aboal et al. (2006a) pointed out that for some elements (Cd, Pb, Zn) local pollution processes are responsible for a substantial part of the variation in concentrations. Schröder et al. (2008) analyzed the same dataset as Pesch and Schröder (2006) and found that the distance from potential emission sources around the SS was one of the main factors influencing metal bioconcentration in mosses. This was confirmed for a French survey by Holy et al. (2009). Kleppin et al. (2008) ranked the distance from housing estates, federal roads and industrial areas within the ten most relevant predictors of metal accumulation in mosses.

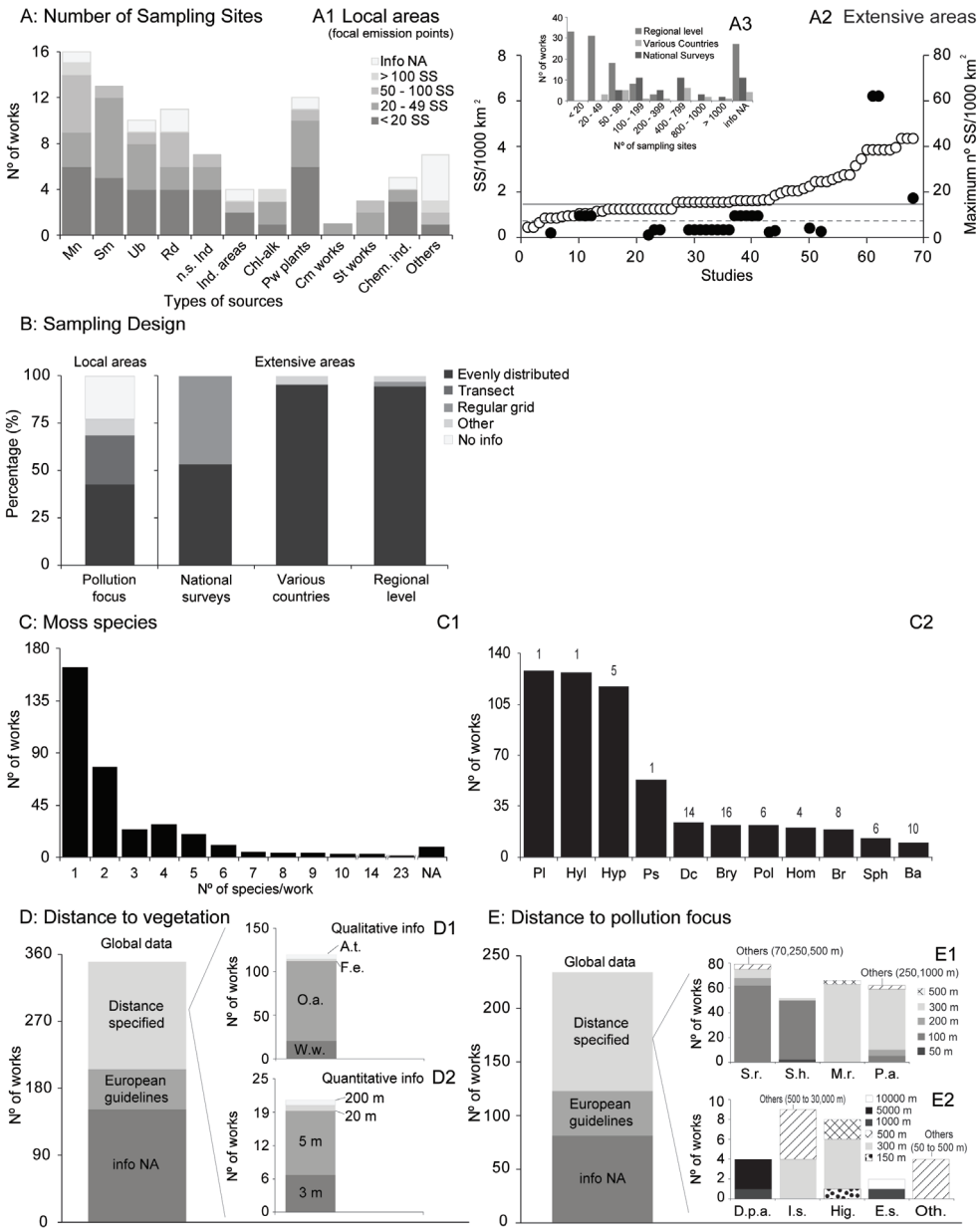


Figure 1: Graphs summarizing the information obtained from the literature reviewed for sections 2.1, 2.2, 2.4, 3.1 and 3.2 of this paper. A) Section 2.1, number of sampling sites (SS); A1) Number of SS used in biomonitoring studies focused on local areas (i.e. point sources of emission). Each bar represents the number of papers for each range of SS and type of emission source studied; Mn: mines, Sm: smelters, Ub: urban areas, Rd: roads, n.s. Ind: non specified industries, Ind. Areas: industrial areas, Chl-alk: chlor-alkali plants, Pw plants: power plants, Cm works: cement works, St works: steelworks, Chem. ind.: chemical industries; Others, including waste dumps, thermometer factories, etc.

Info NA: information not available; A2) SS densities (nº of SS/1000 km^2), ordered from lower to higher (white dots; left Y axis), in studies carried out in extensive areas, including national surveys and studies involving various countries. For studies in which additional sampling was carried out, maximum densities are shown (black dots; right Y axis). The SS densities recommended by ICP-Vegetation (1.5 and 0.8 SS/1000 km^2) are also shown (solid black line and dashed grey line respectively); A3) Number of papers in which the number of SS is specified for regional studies, national surveys and surveys involving various countries. B) Section 2.2, sampling design. Percentage of papers that describe the different sampling designs for both local and extensive studies (the latter including national surveys, surveys involving various countries and regional studies). Other: i.e. radiating, circumferences, etc. C) Section 2.4, moss species; C1) number of different species used per study; C2) most common moss genera (found in more than 10 works), and number of works in which they have been found. The numbers above the histogram bars represent the number of different species found for each of the genera; Pl: *Pleurozium*, Hyl: *Hylocomium*, Hyp: *Hypnum*, Ps: *Pseudoscleropodium*, Dc: *Dicranum*, Bry: *Bryum*, Pol: *Polytrichum*, Hom: *Homalothecium*, Br: *Brachythecium*, Sph: *Sphagnum*, Ba: *Barbula*. D) Section 3.1, distance between SS and vegetation. Number of studies in which the distance between the sampling point and the nearest vegetation was not provided (info NA), followed the European guidelines ("*3 m away from the nearest tree canopy, in forests or plantations primarily in small gaps*") or was specified. The number of studies that mention the distance between SS and vegetation as a qualitative description (W.w.: within woodlands, O.a. open areas, F.e.: forest edges, A.t.: avoiding throughfall – D1) and as a quantitative value (D2), are also shown. E) Section 3.2, distance between SS and source of pollution. Number of studies in which the distance between the sampling point and possible sources of pollution was not provided (info NA), followed the European guidelines ("*at least 300 m from main roads (highways), villages and industries and 100 m away from smaller roads and houses*") or was specified. E1 and E2 represent the number of studies and the distance at which the samples were collected from different pollution sources; S.r.: secondary roads, S.h.: single houses, M.r.: main roads, P.a.: populated areas, D.p.a.: densely populated areas, I.s.: industrial settlements, Hig.: highways, E.s.: emission sources, Oth.: others (i.e. forest paths, helicopter landing areas, etc.).

By analyzing the data from six regional surveys, Carvajal et al. (2010) found that non-compliance with the ICP-V manual recommendations in relation to the distance between SS and roads (paved and non paved roads of different sizes) and buildings (small urban nuclei, isolated houses) did not necessarily distort the regional patterns of metal concentrations. However, the presence of these foci may sometimes lead to increased levels of elements in the nearby SS.

In light of the above-mentioned results, it can be concluded that the recommendations of the ICP-V manual are adequate in relation to distance between SS and roads (300 and 100 m from main roads and smaller roads

respectively), but they are not sufficient in relation to the distance from cities and industries when regional patterns are to be depicted. Thus, SS should be placed at 4 km from industries and 3 km from cities, according to information reported in the literature reviewed. If this is not possible, the potential contribution of local sources to the concentrations of elements in moss samples from specific SS must be studied, as suggested by Carvajal et al. (2010).

3.3 Elevation

The height above sea level (a.s.l.) at which moss samples are collected may be important because elevation determines several environmental factors that may, in turn, affect both the atmospheric deposition of pollutants and the accumulation capacity of mosses. Thus, elevation (often referred to as altitude) may generate gradients in precipitation levels (although not necessarily; Körner, 2007); the slope and aspect will determine the quantity of precipitation that mosses receive (i.e. inputs of wet deposition) and the time of contact between the precipitation and moss (Gerdol and Bragazza, 2006). Other variables such as temperature, wind and snow cover may also display elevational gradients (Krebs, 2001). The level of dry deposition may also be altered due to differences in wind speed, turbulence and vegetation coverage. Finally, the amount of occult precipitation (i.e. cloud, fog droplets) is strongly dependent on topographic factors such as elevation. According to Herckes et al. (2002), the fluxes in deposition of trace metals by cloud water interception can account for up to 50% of the atmospheric wet deposition (even 76% in the case of Pb; Cini et al., 2002).

However, moss growth and physiology are also strongly dependent on environmental and microenvironmental variables (e.g. temperature, radiation, etc.; Zechmeister, 1995a) that necessarily change with elevation (Körner, 2007). Moss growth affects the time that moss tissues are exposed to atmospheric deposition of elements, and the proportion of old/new tissues will determine the heavy metal uptake capacity of mosses (Boquete et al., 2014). Factors such as temperature also determine the kinetics of physiological

processes, including gains and losses of elements.

The ICP-V manual does not include any recommendations regarding the possible effect of elevation on the concentrations of elements in mosses. However, the recommendation given in previous manuals was to collect samples below the timberline in mountainous areas, according to Zechmeister (1995b). This recommendation is quite vague (the timberline is not always at the same elevation) and is based on the results of just one of the different studies that have dealt with this topic (Table 3, supplementary material). In fact, only 15% of the papers reviewed provide information about the elevation at which samples were collected (Fig. 2A) and the type of information provided is quite variable (Fig. 2A1). In addition, the elevation ranges are very different (varying from wide to very narrow depending on the size of the study area and obviously on the heterogeneity in the topography of the area); globally the elevation ranges between 0 and 2490 m a.s.l.

Holy et al. (2009) analyzed the results of the French national survey and concluded that elevation was the most significant factor influencing the local variability. However, the effect of elevation was of negligible significance as found in similar analyses carried out by Pesch et al. (2007) and Kleppin et al. (2008) of the data from German surveys. Therefore, the findings regarding the influence of elevation on the variability of moss concentrations are contradictory. This also applies to studies that have analyzed the correlations between the concentrations of elements in mosses and elevation (Table 3, supplementary material). Very few studies (only 10) have carried out this type of analysis and the elevational ranges considered are not usually comparable. Moreover, some authors removed data points that they considered outliers from the dataset (Zechmeister, 1995b, Soltés, 1998 and Schilling and Lehman, 2002). In the study by Schilling and Lehman (2002), inclusion of the outliers in the analysis yielded a positive significant correlation for Pb and elevation, while the correlation was no longer significant when the outliers were excluded.

The contradictory results of the above-mentioned studies have been attributed to the fact that elevation is a rather simple variable to determine

but that some important variables display more or less steep gradients with elevation (i.e. they act as covariables: amount of precipitation, vegetation, air density and temperature, etc.). Some of these covariables may display local or regional variations due to the specific conditions in each area (e.g. level of precipitation) (Körner, 2007), so that the joint effect of two or more of these variables, together with the possible local/regional variability would alter the results and make their interpretation much more difficult (Schröder et al., 2008). Furthermore, none of the previous studies enable us to conclude whether elevation or its covariables (or their combination) alter the patterns of atmospheric heavy metal deposition or they actually affect the trace element uptake capacity of mosses. Therefore, we conclude that further, more appropriately designed research is required in order to assess how elevation affects the atmospheric deposition of heavy metals and the bioconcentration capacity of mosses. In the meantime, the data obtained from sampling sites at different elevations should be considered with caution.

3.4. Distance from the coast

At least some of the heavy metals that reach moss tissues do so in soluble form (i.e. as metal ions) and their retention will be determined by ion exchange processes at cation exchange sites (CES) (Gjengedal and Steinnes, 1990). The following factors determine which heavy metals will be preferentially retained from those present in the deposited material (i.e. which are the best competitors): i) pH, which will determine the amount of negative charge in the moss surface (i.e. number of CES available) and the chemical form of metals; ii) composition and concentrations of the different elements in the precipitation; iii) physicochemical properties of each metal (i.e. ionic radius and covalence index) (Nieboer and Richardson, 1980); and iv) contact time; i.e. the time needed for efficient retention of elements may increase as the concentrations in the solution decrease (Holmberg, 2006).

Sea spray has been demonstrated to be an important source of Na, K, Mg, Ca, Sr, Ba, etc. to the earth's surface in areas under the influence of these aero-

sols (Whipkey et al., 2000). In a regional moss survey, Aboal et al. (2006a) observed a steep gradient of Na, with high concentrations at coastal sites that decreased steadily towards the interior. In coastal regions in Norway, the levels of Na, Mg, K, Ca and Cl in precipitation were between 3.5 and 12 times higher than the average levels of these elements in the whole country (Gjengedal and Steinnes, 1990). Thus, taking into account that the marine factor will significantly affect the composition of precipitation, and that the composition will determine the preferential uptake/retention of some elements over others, the distance between the moss SS and the coast should be considered in biomonitoring studies.

The ICP-V manual does not mention the precautions that should be observed in relation to the effect of the sea spray on the concentrations obtained. This may explain why 93% of the papers reviewed also do not mention the distance of the SS from the coast. However, Gjengedal and Steinnes (1990) have already demonstrated that Cu and Zn uptake in mosses decreased when the moss was incubated in solutions with high concentrations of sea-salt cations (relative to the usual concentrations in precipitation). Steinnes (1995), stated that the marine factor significantly affected the concentrations of some elements in mosses, and that this effect was negative for Mn and Zn, and positive for Br, Se and Sr. Finally, Kleppin et al. (2008) reported that the concentrations of Al, As, Ba, Ca, Cd, Cr, Cu, Fe, K, Mn, Pb, Sb and Ti were low at sites close to the sea, while those of Na, Mg and V were positively correlated with the distance to the sea due to sea-spray effect and subsequent ionic competition.

In one study, the marine factor, in terms of distance to the sea, was ranked as one of the main factors determining the variability in the accumulation of elements in moss tissues (Kleppin et al., 2008). However, this result was not consistent with the results of similar analyses carried out by Schröder et al. (2008) and Holy et al. (2009). Nonetheless, it seems that sea spray has a significant effect on the concentrations of elements in mosses and may be species specific, as demonstrated by Ashmore et al. (2000). These authors established the correlations between the concentrations of Cd, Cu,

Pb and Zn, and those of Mg and Na in moss samples by analyzing data from the 2000 national moss survey in the United Kingdom. They found that the concentration of Na, in particular, was significantly negatively correlated with the concentrations of Cd, Cu, Pb and Zn in *P. schreberi*, but that the correlation was only significant for Cd in *H. splendens*. Taking into account that this effect cannot be avoided (as samples must be collected in areas under the influence of sea-salt), the results of moss surveys must be interpreted with caution.

3.5. Representativeness of sampling sites

When a moss biomonitoring network is used for carrying out surveys at different times, the SS should be maintained in the same locations for valid comparison of the data obtained. However, changes in land use or in the abundance of the species used may preclude use of the same sites. Therefore, a standardized protocol must stipulate the maximum distance that a SS can be moved between surveys to be considered as the same SS as in the previous survey. According to the ICP-V manual, "*to enable comparison of the data from this survey with previous surveys, it is suggested to collect moss samples from the same (or nearby, i.e. no more than 2 km away but with the same biotope conditions) sampling points as used in the most recent surveys*". However, we did not find any justification for application of this distance in the literature reviewed. Nonetheless, Fernández et al., (2007) collected 36 paired samples of *P. purum*, with a distance of approximately 1 km between the samples of each pair. The distribution of the differences of these paired samples for the studied elements (Al, As, Be, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, V and Zn) were grouped around zero and normally distributed. Markert and Weckert (1989a,b) collected 9 samples of *Polytrichum formosum* from SS located 1.41 km apart; these showed some variability, which was defined as deviations from the average value and varied between ca. 20-70% for Al, Ba, Ca, Cd, Cr, Cu, Fe, K, Mg, Ni, Pb, Sr, Ti and Zn. Taking these findings into consideration, we can conclude that in the absence of small scale point sources of contamination that generate strong contamination gradients (section 3.2),

the SS can be moved a distance of up to 1 km without yielding large variations in the concentrations of elements. At this distance, the variability due to regional contamination gradients will be negligible.

3.6. Dimensions of the sampling sites

The dimensions of the SS from which the subsamples of moss are collected for making composite samples (section 3.7.) must be established. The ICP-V manual states that "*It is recommended to make one composite sample from each sampling point,[…], if possible, collected within an area of about 50 x 50 m*". Other suggestions include that made by Cenci (1998), who recommend different sizes of SS (i.e. maximum area less than 50 m^2, which may be reduced to 1 m2 for compact moss mops). However, again, none of the studies reviewed justify the use of these sizes. The dimensions of the SS used in the papers reviewed are summarized in Fig. 2B. Although the size of the SS is frequently overlooked, the information provided shows that it is highly variable, sometimes even within the same study. The most common size used was 2500 m^2 (55% of the total, Fig. 2B1 and 2B2), which corresponds to the size recommended by the ICP-V manual. The shape of the SS is almost always a square (Fig. 2B3).

The SS should be established in open areas or in forest clearings (see section 3.1). In the latter case, fulfilment of the required distance to the trunks or the shade effect of vegetation outlined in section 3.1, together with the size of the SS, will make it difficult to find forest clearings of the required dimensions. As pointed out by Varela et al. (2010) for a distance of 3 m from the nearest tree and a SS of 50×50 m, the area of a circular clearing should be approx. 0.5 ha; if the height of the vegetation is 20 m, the SS should be located at least 40 m away from trees, which would correspond to a circular clearing of around 2 ha. Difficulties in encountering forest clearings of this size maybe one of the reasons why the recommendation of 50 x 50 m has not been fulfilled in almost one half of the published papers.

We only found one study in which the authors attempted to establish the optimal size of the area to be sampled when applying the moss technique

(Varela et al., 2010). These authors established the size of the SS on the basis of the spatial dependence of the metal concentrations (As, Cd, Cu, K, Hg, Ni, Pb, Se and Zn) in subsamples of *P. purum* collected in four SS. In most cases, there was no spatial structure in the data, or this was fitted to a linear model so that the size of the SS could not be delimited. In a few cases, the spatial structure was fitted to a spherical model and thus, on the basis of the range of autocorrelation (by selecting the most restrictive value of the range), the diameter (Ø) of the SS must be greater than 16 m. Therefore, the area of the SS could be established from a circular area of Ø>16 m (i.e. minimum area of 201 m^2, which constitutes an open space whose dimensions would be easier to encounter) to large sizes such as 50×50 m.

3.7. Number of subsamples

The moss sample should accurately represent the metal concentrations in the SS, including the variability corresponding to the differences among the subsamples. This is an important point as it determines the similarity between the concentrations obtained and the distribution of the mean concentrations of the elements within the SS. Furthermore, it has been suggested that this variability is not related to the magnitude of pollution (Wolterbeek et al., 1996; Wolterbeek and Verburg, 2002). The ICP-V manual suggests collection of between five and ten subsamples (ten for POPs) at each SS. However, the subsample must also be defined. Aboal et al. (2006b) define a subsample as "1 g dry weight (approx.) collected within a 25 cm radius of a node selected at random from a 1 x 1 m grid placed in a SS" (1 g d.w. is equivalent to 20±2 green parts shoots of *P. purum*). When the use of a regular sampling grid and the selection of points at random within the SS is not possible, these authors recommended collection of scattered subsamples separated by 1 m (avoiding an aggregated distribution) within the SS. Zechmeister et al. (2003) define a subsample as all the stems lying within a PVC-tube (Ø = 10 cm) placed over moss plants where ground cover is 100%. Schilling and Lehman (2002) applied the same method, but with a tube of diameter 7 cm. Secondly, the number of subsamples needed to include the

local variability of the concentrations within the SS must be established. Selection of the number of subsamples in the literature is shown in Fig. 2C. Half of the papers reviewed do not give information about whether subsamples were collected, and the others did not specify the number of subsamples. The range recommended by the European guidelines was the most common option (i.e. 5 - 10 subsamples, Figs. 2C1 and 2C2).

Some authors have studied the local variability within a SS by collecting subsamples and analyzing them separately. This variability has been determined in different ways. Thus, i) Rühling (1994a) reported that the local variability reached a maximum value of between 10 and 20%; ii) Kaasik and Liiv (2007) calculated the local variability (25 moss subsamples of *P. schreberi* and *H. splendens* in three SS) as twice the standard deviation plus the error of the analytical method used to obtain the values (maximum for the three sites): Cd – 0.054 $\mu g \cdot g^{-1}$, Cr – 0.84 $\mu g \cdot g^{-1}$, Cu – 0.89 μg g-1, Fe – 235 $\mu g \cdot g^{-1}$, Ni – 0.59 $\mu g \cdot g^{-1}$, Pb – 3.0 $\mu g \cdot g^{-1}$, V – 1.11 $\mu g \cdot g^{-1}$, and Zn – 6.95 $\mu g \cdot g^{-1}$; iii) Cenci (1998) obtained very high coefficients of variation (CV) (9 subsamples): 20% for Al, 12% for Cd, 32% for Cr, 13% for Cu, 19% for Fe, 22% for Hg, 7% for Mn, 23% for Ni, 11% for Pb, 21% for Ti, 17% for V and 20% for Zn; iv) Fernández et al. (2002) (50 subsamples of *P. purum* in one SS) also obtained very high CV: 21% for Ca, 42% for Cu, 38% for Fe, 26% for Hg, 54% for Ni and 38% for Zn; v) Aboal et al. (2006) (50 subsamples of *P. purum* in three SS) reported the following CV (maximum of the three sites): 41% for As, 33% for Cd, 37% for Cu, 37% for Hg, 37% for K, 24% for Ni, 24% for Pb, 39% for Se and 39% for Zn; and vi) Sucharová and Suchara (1998) (10 subsamples of *P. schreberi*, *P. purum* and *H. cupressiforme* at 20 SS) calculated the variability in analytical data as a percentage of the relative standard deviation: 13.5% for Al, 13.7% for As, 9.5% for Cd, 14.6% for Co, 18.2% for Cr, 10.2% for Cu, 13.2% for Fe, 9.7% for Mo, 12.8% for Ni, 10.5% for Pb, 7.1% for S, 13.4% for V, and 10.1% for Zn. In general, the intra-SS variability corresponding to the differences between the subsamples is usually very high; indeed, Sucharová and Suchara (1998) identified this as the main source of variability in the analytical data.

The number of subsamples required for inclusion of the intra-SS variability can be calculated by three different approaches, according to the type of experiment: i) by applying bootstrap procedures; ii) by calculating the error of the mean; and iii) by calculating the number of subsamples required to obtain significant differences in the mean concentrations between two SS. Wolterbeek and Verburg (2002) collected 25 moss subsamples at one SS and analysed each separately. They used bootstrap procedures simulating n=5 subsamples and concluded that 5 subsamples yielded reasonable results (consistency between subsampling and local populations for As, Br, Cr, Cs, Cu, Fe, K, Mg, and Zn, in 90% of the trials). Fernández et al. (2002b) calculated the number of subsamples, assuming an error of 20% of the mean, and obtained the following results: 4 for Ca, 17 for Cu, 14 for Fe, 7 for Hg, 28 for Ni and 14 for Zn. These authors suggested collection of at least 30 subsamples. Finally, Aboal et al. (2006b) concluded that collection of 30 subsamples was required to determine significant differences in the mean concentration between two SS (one uncontaminated and a slightly contaminated site) for As, Cd, Cu, Hg, K, Ni, Pb, Se and Zn.

Fernández et al. (2002b) also studied the effect of the difference in weights of the subsamples in a composite sample on the measurement of element concentrations, and they concluded that this factor is of little importance (up to 2.5%); nevertheless, this error could be removed by using well-defined subsamples. According to Wolterbeek et al. (1996) analytical variability contributes very little to local variability. Nevertheless, this is an unavoidable source of error; the concentrations of metals and metalloids in the subsamples corresponding to a SS are not usually normally distributed and are generally greatly skewed to the right (Fernández et al., 2002b; Aboal et al., 2006). Analysis of a composite sample is the equivalent of calculating an arithmetic mean; however, if the population of samples is not normally distributed, then the error produced and could generates differences of >10% (Fernández et al., 2002b). The distribution of subsamples within a defined SS area will determine the degree to which the heterogeneity of that surface is included in a single sample (Fernández et al., 2002b).

As the error associated with the possible lack of normality in the distribution of the concentrations of elements in the subsamples cannot be avoided, harmonization of the methodological aspects addressed in this section should focus on the following: i) establishment of a standard definition of "subsample"; and ii) the definition of the minimum number of subsamples that should be collected per SS. In regard to the first point, we recommend the following definition of a subsample: "all the stems lying within a PCV-tube of diameter that would yield 1 g d.w. of moss material". The diameter of the tube may slightly vary depending on the size of the species and its pattern of growth (e.g. more or less compact mats). However, a diameter of 25 cm would probably be suitable for the most common pleurocarpous mosses used in biomonitoring studies. Regarding the number of subsamples, the number recommended in the ICP-V manual (5 to 10 subsamples) does not seem sufficient to reflect the intra-SS variability. Therefore, and on the basis of the results reported in the literature reviewed, collection of a minimum number of 30 subsamples distributed homogeneously within the SS is recommended.

3.8. Quantity of sample collected

The number and size of the combined subsamples will determine the weight/volume of the composite sample. Wolterbeek and Bode (1995) suggested that the total mass of sample that should be processed to yield a homogenous analytical sample may be a key point in biomonitoring surveys. The ICP-V manual states that the amount of fresh moss needed for metal analysis is about 1 L. Previous moss monitoring manuals (Rühling, 1994b) recommend collection of around 2 L of moss material. Cenci (1998) recommends collecting between 20 and 50 g of moss. Most of the papers reviewed do not state the quantity of material collected (Fig. 2D). However, highly variable amounts are reported in the remaining studies. Once again, the most common option was that recommended in the ICP-V manual. Only 21% of the papers specified whether dry or fresh weight was used.

According to Aboal et al (2006b), the amount of sample collected must be

sufficient for all the required analyses, with a portion left over for posterior storage. Thus, if 10–15 g d.w. of moss are required for analysis of dioxins and furans (e.g. Abad et al. 2003), 5 g d.w. for PAHs (e.g. Gerdol et al. 2002b) (actually these quantities could be decreased to less than 5 and 1 g respectively), and approximately 0.5 g for the analysis of metals and metalloids, at least 7-20 g d.w. of material would be required for a single analytical determination, if repetition is not required. Even when only a small amount of composite sample is required for a specific analytical method (e.g. 0.5 g d.w. for heavy metal analysis), the number of subsamples combined should not be less than the estimated sample size (section 3.7). As terrestrial mosses are routinely stored in Environmental Specimen Banks in Finland, Denmark, Sweden, Iceland, Norway, Japan, the United States and Spain (Galicia) (Aboal et al. 2001), the final weight of the sample must also include the amount of moss destined for storage. For example, in a survey with *P. purum*, when the composite sample comprises 30 subsamples (according to section 3.7), the final weight of the sample will be 30 g d.w. of moss (equivalent to a volume of approx. 2 L of moss before the selection of material, approx. 600 green parts shoots of P. purum), which is consistent with the recommendations of Rühling (1994b). The moss should be placed in bags made from a material that does not contain the elements under study (e.g. in polythene or paper bags).

3.9. Sampling precautions

According to the ICP-V manual, researchers should wear plastic gloves (or place plastic bags over their hands) when collecting samples. Only powder free vinyl examination gloves should be used as the powder may contain heavy metals. Smoking is also forbidden during the sampling and further sample handling. This is because tobacco smoke contains large amounts of heavy metals (i.e. As, Cd, Cr, Ni, Pb and Zn) and other substances for which mosses are used as biomonitors, such as benzo[a]pyrene benzo[a]anthracene, PCDDs, PCDFs, etc. (IARC, 1985; USDHHS, 1986; EPA, 1992).

3.10. Sampling period

In the design of moss biomonitoring studies, the temporal variability in the concentrations of elements in moss may be problematical in relation to representativeness of the samples obtained. The ICP-V manual specifies that "*Samples should preferably be collected during the period April - October. In arid regions of Europe it is advised to collect the samples during the wet season. [...] Therefore, it is suggested to sample the mosses in the shortest time window possible*". The sampling periods reported in the literature reviewed are shown in Fig. 2E. Most studies do not state the specific length of this period. The reported sampling periods (Fig. 2E1) are highly variable, with the most common duration being between 2 and 4 months (which is consistent with the previous recommendation). The collection period is also highly variable (Fig. 2E2), and usually comprised different lengths of time between spring and autumn. With respect to the scientific reasons for selecting the sampling period, some authors justified collection of samples in autumn (mid-September and October) as this is assumed to be the end of the growing season (i.e. Gerdol et al., 2002a; Gerdol and Bragazza, 2006; Yurukova et al., 2009; Thöni et al., 2011), which should yield data on metal accumulation during the precedent growth period (Faus-Kessler et al., 2001). However, Bealey et al. (2008) affirm that for measurement of the concentrations of heavy metals in mosses, sampling should be carried out during the growing season and that the actively growing green shoots should be sampled to maximise the pollutant loads. According to Glime (2007), maximum growth occurs in spring and October-December in most temperate bryophytes.

The temporal variability in the concentrations of elements in moss may be seasonal. In those studies in which sampling was carried out over a maximum period of one year (Zechmeister et al., 2003; Leblond et al., 2004; Boquete et al., 2011a), no seasonal variability in the concentrations was usually observed, although with some exceptions (Table 4 supplementary material). These data are affected by the part of moss analyzed (Leblond et al., 2004). Different authors state that sampling can be carried out during a

whole year or during a particular period for the species under study (Thöni et al., 1996; Berg and Steinnes, 1997; Fernández and Carballeira, 2002). However, the literature reviewed reflects problems in the statistical analyses used to reach these conclusions. In these studies the values were grouped for different seasons or sampling times, and between-group differences were explored by use of a Kruskal-Wallis test (Berg and Steinnes, 1997; Fernández and Carballeira, 2002; Zechmeister et al., 2003; Leblond et al., 2004) or Friedman's rank-sum test (Zechmeister et al., 2003). However, lack of significant differences in the metal concentrations between sampling times may be attributable to the small data set and the very rigid calculation of the tests (Zechmeister et al., 2003), as the temporal patterns are obvious (Fig. 2 of the latter study and Fig. 6, for Cd, in Berg and Steinnes, 1997). Moreover, the absence of differences may be due to the inclusion of SS with very different concentrations of elements, the variation in which may mask the differences in relation to sampling time, as demonstrated by Couto et al. (2003). The existence of seasonal differences can only be established when data series spanning more than one year are available, as shown by Markert and Weckert (1993) and Couto et al. (2003) (Table 4 supplementary material), as the only correct way of showing their existence is by studying temporal correlations.

Independently of seasonal changes, there is an enormous amount of temporal variability, which introduces a large degree of variability in the concentrations obtained. The data obtained at a given time in sampling survey are therefore not representative of the mean concentrations corresponding to that SS for a time period (i.e. annual, etc.). Thus, Markert and Weckert (1989b) observed annual variations in the concentrations of heavy metals of up to 80% in some cases. Likewise, Real et al. (2008) reported pulses of contamination of short duration in which differences in Hg concentrations ranged from approximately 500 to 3200 $ng \cdot g^{-1}$ in an interval of one month; Leblond et al. (2004) reported than in an interval of 1 month, the concentration of Hg varied between the minimum and maximum values obtained during a year. According to Boquete et al. (2011a), the percentage error in

determinations made in a period of 3-4 days reached 345% for Al, 417% for Cd, 145% for Cu, 520% for Cr, 368% for Fe, 404% for Hg, 57% for K, 94% for Na, 393% for Ni, 1058% for Pb, 221% for V and 127% for Zn. Different authors have compared this source of error with that associated with spatial variability (Markert and Weckert, 1989b), the variability between subsamples (Zechmeister et al., 2003), and the analytical variability (Markert and Weckert, 1989b; Boquete et al., 2011a). All of these authors concluded that these sources of error are very low in comparison with the temporal error.

According to Markert et al. (1999), mosses should be collected in the survey area in as short a time as possible, preferably when the monitor contains high levels of the xenobiotics concerned. Zechmeister et al. (2003) concluded that the sampling period should be as short as possible and samples collected in different seasons (summer, autumn) should not be compared in national programmes. Both these recommendations and those outlined in the ICP-V manual are problematical, for the following reasons: i) if seasonal patterns exist, the maximum and minimum values will usually be separated by 6 months (Table 4 supplementary material) - the period between April and October covers 6 months and the sampling may coincide with maximum or minimum concentrations; ii) as explained by Leblond et al. (2004) regarding the temporal variability, the moments at which the maximum and minimum values occur will depend on the particular environment and will therefore not be the same in large areas; iii) independently of the existence of seasonal patterns, the problem of the temporal representativeness of a single sample is totally overlooked; iv) sampling over large areas requires several weeks or even months. It is therefore impossible to obtain synchronized data, because if the variation in the concentrations occurs within a matter of days, comparison of samples collected several days apart will not be valid (Boquete et al., 2011a).

Although the errors associated with sampling (see sections above) and chemical analysis may be minimized, the error associated with the temporal variability is almost inevitable and uncontrollable. According to Boquete et al.

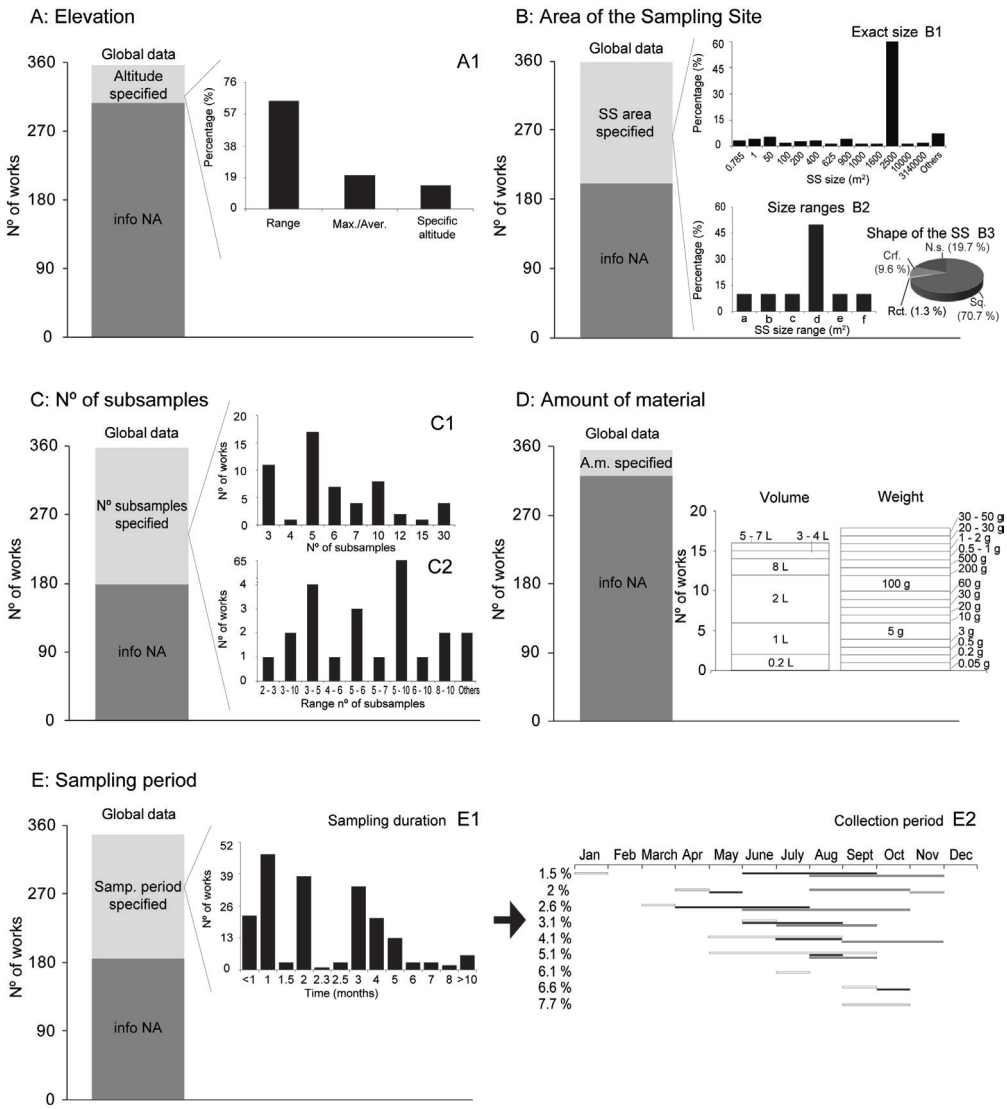


Figure 2: Graphs representing the information obtained from the literature reviewed for sections 3.3, 3.6, 3.7, 3.8, 3.10 of this paper. A) Section 3.3, elevation. Number of papers in which the elevation at which samples were collected was not provided (info NA) or was specified (elevation specified); A1) Percentage of papers in which elevation was taken into account either by giving the range of elevation at which samples were collected, the maximum or average elevation (Max./Aver.) or the specific elevation for each SS. B) Section 3.6, area of the sampling site (SS). Number of papers in which the area of the SS was not provided (info NA) or was specified (SS area specified); B1) Percentage of papers in which the exact size of the SS is given (m^2) for each of the sizes found; B2) Percentage of papers in which the size of the SS was within a range (a: 20-30 m^2, b: 50-200 m^2, c: 380-7850 m^2, d: 400-2500 m^2, e: 900-2500 m^2, f: 1225-2500 m^2); B3) Percentage of papers in which the shape of the SS was specified (Sq.: square, Rct.: rectangle, Crf.: circumference) or was not

specified (N.s.: not specified). C) Section 3.7, number of subsamples. Number of papers in which the number of subsamples was not provided (info NA) or was specified (Nº subsamples specified); the number of papers in which the exact number of subsamples was provided (C1), and in which the number of subsamples was within a range (C2) for the different options found, are also shown; others: papers in which the number of plant shoots collected at each SS was specified (20 and between 50-100 shoots per m^2 respectively). D) Section 3.8, amount of material collected. Number of papers in which the quantity of material collected was not provided (info NA) or was specified (A.m. specified: Amount of material specified). When specified, the amount of material was expressed by volume (L: litre) or by weight (g: grams). The number of papers in which the different options regarding volumes and weights were specified are also shown. E) Section 3.10, sampling period. Duration of the sampling period (in months – E1) and percentage of times that samples were collected for each moment of the year (E2 – the horizontal bars drawn within each percentage correspond to the different collection periods that showed the same frequency of appearance).

(2011a), large numbers of samples should be collected in each SS over time to provide temporally representative data, and obviously the accuracy of the characterization will increase with the number of samplings. However, scientific convention must be balanced by the reality of practical and economic considerations (one great advantage of the moss biomonitoring technique is the low cost of the method). Thus, Couto et al. (2003) concluded that the optimal sampling design is two sampling events separated 6-7 months, but without any restrictions on the time of year that the sampling is carried out; however, this reduces the improved temporal representativeness. Therefore, as pointed out by Boquete et al. (2011a), the results obtained must be interpreted as qualitative or semi-quantitative data on air quality, rather than as absolute data with minimal temporal representativeness and a high degree of associated uncertainty. According to Real et al. (2008), another possible way of monitoring the seasonal changes in moss concentrations is the complementary use of regional sampling networks sampled at relatively low frequency (each 1, 2, 5 years etc.) and some additional SS sampled at high frequency (weekly-monthly). Combined interpretation of the data provided by both types of survey will provide more accurate information about the air quality as this type of system enables detection of peaks in emissions of the pollutants considered.

4. Cleaning and storing

A general recommendation according Wolterbeek and Bode (1995) is that if several laboratories are participating in sample handling, some samples should be distributed as quality controls at the beginning of the cleaning and storing steps.

4.1. Sample treatment

Samples may be cleaned immediately after sampling (e.g. Halleraker et al., 1998; Äyräs et al., 1997; Reimann et al., 1997) or even in the field (Aboal et al., 2008), as this prevents remobilization of heavy metals within the moss shoots associated with the growth in the laboratory (Brown and Brown, 1989) and an increase in element concentrations during storage (Wells and Brown, 1990). Researchers usually collect a large volume of moss, place this in a sampling bag and store the bag for an undetermined number of days or weeks, until the samples are finally cleaned in the laboratory. The ICP-V manual states that "*If the samples cannot be cleaned straight after sampling, they should be put into paper bags and dried and stored at room temperature (20-25 ºC) until further treatment. Alternatively, samples can be deep-frozen*". Only a very small percentage of studies provide information on this aspect (Fig. 3A). Three main options for storage of samples during this time period are reported (Fig. 3A): i) in paper bags and air-dried and stored at room temperature (20–25°C); ii) frozen (-18 – -30°C); and iii) in the fridge (4–5°C) if the samples will be cleaned within 2 weeks of collection. Aboal et al. (2008) found significant and not negligible differences between the concentrations of elements (i.e. Al, As, Cd, Cr, Cu, Fe, Hg, K, Ni, Pb, Se, V and Zn) measured in *P. purum* on applying these 3 methods.

Taking into consideration the above comments, samples should be cleaned immediately after sampling. If this is not possible, samples should be stored in the fridge in sealed plastic bags to avoid contamination, to ensure the integrity of the plasma membranes and to prevent the detachment of adhered particles. The samples should not be stored for more than two weeks.

4.2 Selection of material

Selection of the material is a key step in processing the moss and is one of the main problems in moss biomonitoring (Leblond et al., 2004) because of the differences in metal concentrations in different parts of the moss shoots. Such differences have been detected i) on comparing the basal and apical parts of the shoots in different species (i.e. Stryjewska et al., 1994, and studies compiled by Leblond et al., 2004), ii) in studies in which metal uptake was monitored (e.g. Brown and Brown, 1989), and iii) in a study of the relationship between metal uptake and size of the shoots (e.g. Okland et al., 1997). However, the data reported in the literature are contradictory as regards which metals are preferentially accumulated in older tissues and in younger parts.

The ICP-V manual specifies that "*just the green and green-brown shoots from the last three years growth are included. Brown parts should not be included, even if the green parts only represent the last two to three years of growth*". However, this recommendation is very vague and its application is restricted to some moss species in which it is possible to determine the age of the segments due to the presence of well-defined annual segments (*H. splendens*) or variations in the space between branching zones (*A. abietina*) (Zechmeister et al., 2003). In most of the species used in biomonitoring, e.g. *P. schreberi*, *P. purum*, *H. cupressiforme*, it is impossible to distinguish the last two to three years growth. The lack of definition is reflected in the enormous variability reported in the literature consulted (Fig. 3B). The most usual option is the use of segments or growth increments of a determined age (Fig. 3B1, with different variants (e.g. green parts, green and greenish brown parts, the whole shoot, the aboveground parts, the living parts, and a selected apical length) (Fig. 3B2). Many of the papers reviewed applied this type of definition for species such as *P. schreberi*, *P. purum* and *H. cupressiforme* in which annual increments are not actually distinguishable. The following are some of the other most commonly used options (Fig. 3B1): green parts, green and greenish brown parts, whole shoot, aboveground parts (to avoid soil

contamination), live parts, and a selected apical length (including different lengths as shown in Fig. 3B2).

The material should be sorted on a clean surface (e.g. made of PVC or ceramic material), and the selected material should be cut with plastic or ceramic scissors or separated manually (with the handler wearing gloves as described in section 3.9) in accordance with Cenci (1998). Regarding selection of the material according to its colour (i.e. green parts or green and greenish brown parts), it is difficult to find an objective means of establishing the limit between the parts that are used and those that are discarded. Regarding selection of the length of the apical portion - although it is often assumed that these parts will represent or correspond to a certain time period (e.g. Ermakova et al., 2004; Frontasyeva et al., 2004; Barandovski et al., 2008), this is an error as the growth rates vary hugely between species and between sites in the same region for the same species (Boquete et al., 2014). Moreover, selecting specific segments from species with well defined annual segments does not ensure that the concentrations of pollutants will represent the material deposited during a specific period of time as age-related differences may also be observed in this type of species (i.e. *H. splendens* as indicated by Brown and Brümelis, 1996).

As none of these recommendations presented in the ICP-V manual enable standardization of the time of exposure of the portion of moss shoot selected for analysis, nor the age of the mentioned portion, we recommend selecting the green parts of moss shoots (i.e. living tissues). This will prevent the establishment of any temporal relationship between the concentrations found in mosses and the atmospheric deposition, but will homogenize the physiological status of the tissues analysed and minimize age-related differences in the cation uptake/retention capacity of tissues (Boquete et al., 2014).

4.3. Sample cleaning

The ICP-V manual indicates that the moss shoots should be cleaned to remove any adhered material (i.e. dead material and attached litter) before

chemical analysis. Thirty two per cent of the papers reviewed do not give any information about whether the samples were cleaned. Most of the studies that do include the information (75%) use the sample cleaning method proposed by the ICP-V manual. Nevertheless, in some studies the objective was to remove the particles deposited on the moss surface, which required a second washing process. According to Markert et al. (1999), the main reason for removing external particles is to assess phytotoxicity and availability to plants, and therefore any deposited material adhering to the surface of the monitors must be removed prior to analysis. This practice should only be carried out for these purposes and should not be routinely used in the moss monitoring technique.

Different methods have been proposed for the removal of particles: i) washing; ii) use of a nitrogen jet (Ducceschi et al., 1999; Spagnuolo et al., 2013) or air jet (Schintu et al., 2005; Sardans and Peñuelas, 2005); iii) carefully brushing the moss shoots (Sardans and Peñuelas, 2005); iv) viewing the samples under a binocular microscope to aid in removal of particles (Gasparon and Matschullat, 2006); and v) the use of ultrasound (Spagnuolo et al., 2013). The efficacy of removal of particles has been found to be low (Aboal et al., 2011; Spagnuolo et al., 2013). Of these methods, the most common choice is to wash the samples (17% of the all papers reviewed). Irrespectively of the type of washing procedure used, there is no doubt about the effect of washing on the concentrations of elements in moss, as pointed out by different authors for different species (i.e. *Entodon scheberi*, *H. cupressiforme*, *P. schereberi* and *P. purum*) and numerous elements (i.e. Al, As, Cd, Cr, Cu, Fe, Hg, K, Mn, Ni, Pb, Se, Tl, V and Zn) (Stryjewska et al., 1994; Türkan et al., 1995; Dmowski and Badurek, 2002; Fernández et al., 2010). Exceptionally, Aničić et al. (2007) did not find differences for *Brachythecium* sp. and *Eurhynchium* sp., although the statistical approach used by these authors does not seem appropriate. In conclusion, the differences between washed and unwashed samples were of high magnitude and therefore this aspect should not be ignored. This, together with the inefficiency of the particle removal implies that the sample cleaning should be restricted to the above-mentioned re-

moval of material that is obviously not part of the moss tissues.

4.4. Sample drying

Moss samples should be dried before being homogenized. The samples should be cleaned and dried as soon as possible after being collected, to prevent the problems described in section 4.1. The ICP-V manual states that "*The samples should be dried to constant weight at 40ºC, which is used as a reference for the calculations. It is recommended to record the drying loss at 40ºC (compared to room temperature) for future reference. […] For mercury, analysis should be conducted on fresh material or material dried at a lower temperature than 40ºC and the determination of drying loss at 40ºC on a separate aliquot is recommended*". According to Cenci (1998), moss should be dried in an oven at 45ºC for 48h (time for 10-20 g deposited in a cylinder of 20 cm of diameter and 10 cm of height); an aliquot of moss from each station, should be dried at 105ºC, for calculation of water loss. This is common practice in the literature reviewed (i.e. Gerdol et al., 2002a; Giordano et al., 2004; Gramatica et al., 2006; Farmer et al., 2010)

Drying at 45ºC minimises losses due to volatilization of elements such as Hg and Pb. Although high temperatures (i.e. >45%) are usually avoided in pretreatment of moss samples for the above elements, no studies have specifically addressed the effect of the drying temperature on heavy metal moss loads. Nevertheless, in a study of the adsorption and desorption of Hg by moss under laboratory conditions, Lodenius et al. (2003) observed strong sorption of Hg to moss even at 60ºC, which indicates that higher temperatures may be able to be used for moss matrixes. Most of the studies reviewed include information about drying (Fig. 3C), with a temperature below 40ºC most common, in accordance with the ICP-V manual. The material is usually dried in an oven, although in a few cases the samples were lyophilized (i.e. Minger and Krähenbühl, 1997; Koch et al., 2000; Allen-Gil et al., 2003).

To minimize the loss of the most volatile elements, we recommend drying moss samples at 40ºC, as outlined in the ICP-V manual. As moss species may

differ in the rate at which they lose water when dried at 40 ºC, we recommend drying an aliquot of each sample at 105ºC (constant dry weight) and expressing the concentrations on a dry weight basis.

4.5. Sample homogenization

The method most commonly used to homogenize moss samples is grinding/milling. Although this step is not strictly necessary, it is important as it increases the analytical replicability (Stryjewska et al., 1994). It also reduces the sample volume, which facilitates posterior storage. Although some authors prefer not to grind the samples, in order to minimize contamination during sample preparation (Steinnes et al., 2011), most studies reviewed include this step (Fig. 3D). It is important that the grinding equipment is made of heavy metal free materials, such as titanium, titanium-niobium alloys or agate (Fig. 3D). Equipment made from steel or tungsten oxide is sometimes used, which may lead to contamination of the samples with heavy metals. In a few cases, the material is ground by hand in a porcelain mortar. It is also important to use mills that do not generate high temperatures during the milling as a result of friction between the sample and the equipment (especially when metals such as Hg, Pb or organic compounds are being measured).

Cenci (1998) suggests that the particle size should be less than 125 μm, to ensure good homogeneity of the analytical sample, which usually weighs between 100 and 200 mg. However, this recommendation is rarely met and in the literature reviewed, the sizes ranged between 63 μm (Migaszewski et al., 2002) and 2 mm (Davis et al., 2007), with a particle size of 200 μm being the most common (e.g. Ratcliffe, 1975; Suchara and Sucharová, 2004; Lee et al., 2005; Chen et al., 2010). Liquid nitrogen is sometimes added when the material is ground (i.e. Beckett et al., 1982; Wolterbeek and Verburg, 2002; Zechmeister et al., 2005; Krommmer et al., 2007; Zechmeister et al., 2008), although this precludes posterior determination of nitrogen. Our experience leads us to suggest the use of tangential mills (with zirconium beads and receptacles) for small samples, to yield particles <10 μm. For large sam-

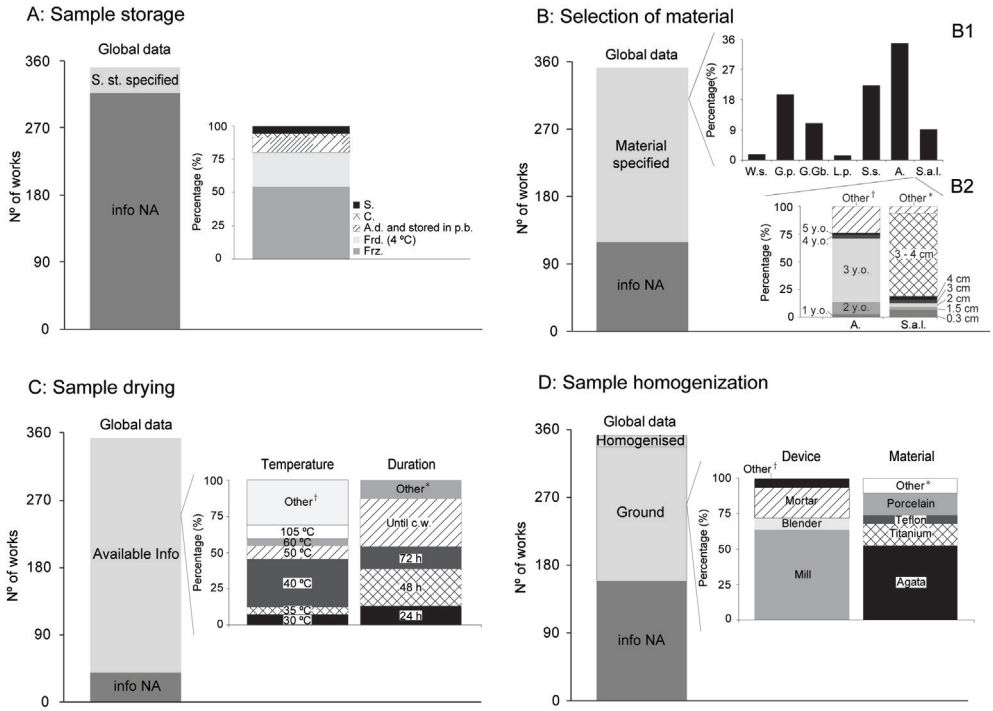


Figure 3: Graphs representing the information obtained from the literature reviewed for sections 4.1, 4.2, 4.4, 4.5 of this paper. A) Section 4.1, sample storage. Number of papers in which the sample storage method was specified (S.st. specified: sample storage specified) or was not specified (info NA). The percentage of papers that used each of the options is shown; Frz.: frozen (i.e. -18, -20, -25ºC, etc.), Frd.(4ºC): fridge at 4ºC, A.d. and stored in p.b.: air dried and stored in paper bags, C.: cooled, S.: sealed. B) Section 4.2, selection of material. Number of papers in which the material (part of the moss shoot) selected for analysis was mentioned (Material specified) or was not specified (info NA); B1) Percentage of papers in which each of the different parts of moss shoots were used; W.s.: whole shoots, G.p.: green parts, G.Gb.: green and greenish-brown parts, L.p.: living parts, S.s.: specific segments (i.e. upper 3 annual segments, last 3 years growth, increments of the previous 2 years, etc.), A.: ages, S.a.l.: selected apical lengths; B2) Percentage of papers in which a specific age or a selected apical length were selected. Other†: 1-2, 1-3, 1-5, 2-3 and 2-5 years old segments; Other*: 1.5-2 and 1-3 apical cm of moss shoots. C) Section 4.4, sample drying. Number of papers that provided information about the drying step, or not (info NA). The percentage of studies in which samples were dried at the different temperatures and times (duration) is also shown (Other†: room t^{a}, 44, 70, 80 ºC, etc.; Other*: 2, 3, 36 hours, overnight, etc.); Until c.w.: until constant weight. D) Section 4.5, sample homogenization. Number of papers that provide information about the homogenization step (milled or homogenized), or not (info NA). The percentage of papers in which the different devices and materials used are also specified (Other†: vessels, capsules, etc.; Other*: wolfram, plastic, steel, etc.).

ples, use of ultracentrifugal mills is recommended, although larger particles are produced (e.g. for titanium-niobium receptacles and 200 μm rings, the particle size distribution for *P.purum* is as follows: 0.6% >200 μm; 12.2% between 100 and 200 μm; 40.5% between 50 and 100 μm and 46.4% < 50 μm; for *H.cupressiforme* the distribution is 1.4% >200 μm; 34.0% between 100 and 200 μm; 10.5% between 50 and 100 μm and 54.1% < 50 μm). Dried moss tissue is rather elastic, which may make it difficult to grind in some types of mill (e.g. orbital).

4.6. Storage

The receptacle in which the homogenized moss is stored should be long-lasting and allow removal of aliquots of each sample. The headspace should be minimized to prevent loss of elements via volatization. The types of material recommended are glass, quartz and teflon, as some problems have been reported in relation to the use of receptacles made from different types of plastic (Ambe, 1983; Wagner, 1995). A glass or zirconium ball can be placed in the receptacle to homogenize the ground moss (Cenci, 1998). For analysis of inorganic contaminants, samples are usually stored in darkness at between 4 and 20ºC. Depending on the study objectives in relation to the contaminants considered, samples may be stored by dry freezing at between -20 and -80ºC or by cryogenic freezing at between -80ºC and -196ºC.

Final remarks and conclusions

The consistency of the protocols reported in the literature reviewed (those focused on use of the moss technique in extensive areas) with the protocol outlined in the ICP-V manual protocol was assessed. Thus, the articles reviewed were qualified according to 13 aspects, with 0 - 2 points awarded for each aspect. The scores therefore varied between 0 (non fulfilment or information lacking of any of the aspects included) and 26 (complete fulfilment of the ICP-V manual recommendations) (Fig. 4). The results of this evaluation showed that none of the studies reviewed completely fulfilled the protocol suggestions. In fact, 88% of the studies were awarded less than half

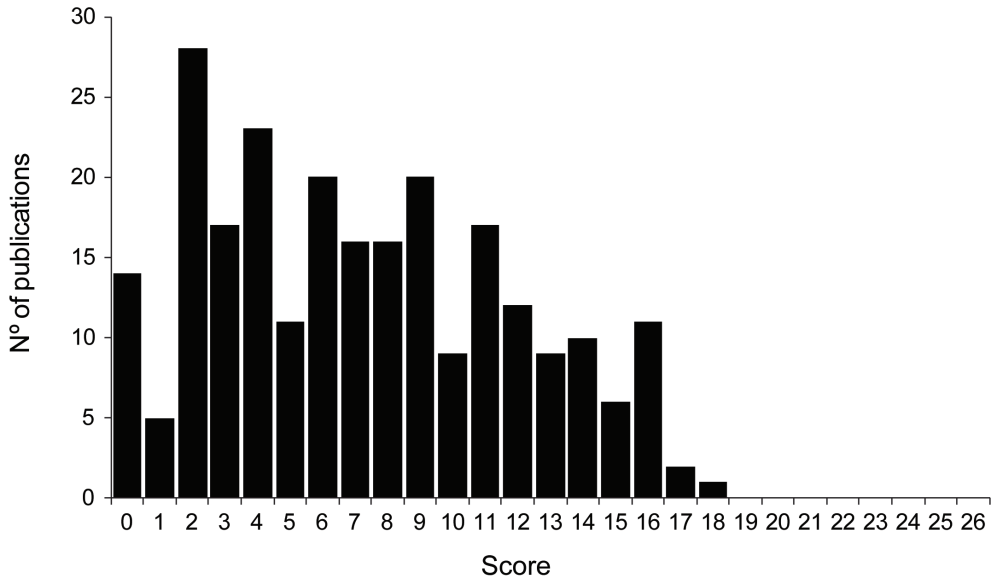


Figure 4: Assessment of the degree of fulfilment of the ICP-V manual protocol in the revised papers (those focused on the application of the moss technique in extensive areas). Articles were qualified according to 13 aspects (from the evaluated in this review) by awarding between 0 and 2 points for each aspect. The scores thus vary between 0 (non fulfilment or information not provided about any of the aspects included) and 26 (complete fulfilment of the ICP-V manual recommendations).

of the maximum score. The low score was sometimes due to lack of information in the paper (e.g. amount of sample collected, samples treatment); in other cases, low score was obtained simply because the authors did not follow the ICP-V manual suggestions (e.g. distance between SS and vegetations, selection of material, etc.). This demonstrates the need for a more detailed, rigorous and clearly defined protocol for application in passive biomonitoring studies with terrestrial mosses. Authors must also include and specify each of the aspects involved when applying the technique to enable inter-study comparison of the results as the lack of this type of information will devalue the results and the conclusions reached.

Taking into account all the information included in this review paper, we conclude that, when stated, the protocol most commonly used in biomonitoring studies regarding the aspects involved in the application of the moss technique is usually that suggested in the ICP-V manual. However, the scien-

tific evidence sometimes contradicts the manual recommendations. Therefore, we propose a new protocol in which each suggestion has been carefully and rigorously compared with the information available in the literature. The proposed protocol is more restrictive than the ICP-Vegetation protocol and very few, if any, of the studies published to date would fulfil the requirements. However, as the steps are much more concisely outlined, application of the protocol will be less subjective and easier to fulfil. Practical and economic considerations have also been taken into account for each decision, and when the effect of any aspect could not be minimised or avoided (e.g. elevation, distance from the coast), caution is recommended in interpreting the results of the biomonitoring studies. The new proposed protocol is summarized in Table 1 of this review paper.

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Table 1: ICP-V manual recommendation, option most commonly used and final recommendation for each of the variables involved in the application of the technique of passive biomonitoring with terretrial mosses. spp/sp: species; H.s.: *Hylocomium splendens*; P.s.: *Pleurozium schreberi*; H.c.: *Hypnum cupressiforme*; P.p.: *Pseudoscleropodium purum*; Ø: diameter; d.w.: dry weight; POPs: persistent organic pollutants; y.o.: years old.

Variable	ICP-V manual	Option most commonly used	Recommendations
1. Pre-sampling aspect			
1.1 Nº of sampling sites (SS)	1.5 or 0.8 samples/1000 km^2	1.5 samples/1000 km^2	1 sample/1000 km^2 (4, 16 or 62 samples/1000 km^2 when more resolution is needed)
1.2 Sampling design	SSs evenly distributed over area	SSs evenly distributed over area	Regular sampling
1.3 Survey frequency	Once every 5 years		As frequent as possible (economic limitations)
1.4 Moss species	1 spp (H.s., P.s., H.c. or P.p.)/when >1 species, calibration required	1 species (P.s., H.s., H.c., P.p.)	No calibration; if more than 1 species is used, qualitative interpretation
2. Field Sampling			
2.1 Distance between SS and vegetation	forest gaps (Ø > 10 m), plantations primarily gaps (Ø > 5 m); 3 m away from tree canopy	Open areas/5 m from trees	y = 23.63 ln(x) - 31.53/when possible 2·Height of the tree (under an angle of 22.5º)
2.2 Distance between SS and pollution sources	300 m from main roads, urban and industrial nuclei/100 m small roads, isolated houses	300 m from main roads, urban and industrial nuclei/100 m small roads, isolated houses	300 m from main roads/100 m from small roads/4 km from industries/ 3 km from cities
2.3 Elevation	No recommendation	Highly variable	Caution in interpretation of results
2.4 Distance between SS and coast	No recommendation	Not taken into account	Caution in interpretation of results
2.5 Representativeness of SS	Same SS or 2 km away but in the same biotope	Not taken into account	1 km away from the original SS

2.6 Area occupied by the SS	50 x 50 m	50 x 50 m	Between 201 (circular area, Ø > 16 m) and 2500 (50 x 50 m) m^2
2.7 Nº of subsamples	5 to 10 subsamples (10 for POPs)	5 to 10 subsamples	Minimum of 30 subsamples
2.8 Amount of sample collected	1 L (2 L in previous manuals)	2 L	30 g d.w. of moss
2.9 Sampling precautions	Use of plastic gloves (powder free)/ no smoking	Use of plastic gloves (powder free)/no smoking	Use of plastic gloves (powder free)/no smoking
2.10 Sampling period	during April – October/shortest time window possible	1 month duration/spring to autumn	2 samplings separated by 6-7 months
3. Cleaning and Storing			
3.1 Sample treatment	Paper bags, dried and stored 20 - 25 ºC/frozen	Frozen	Stored in the fridge in sealed plastic bags (< 2 weeks)
3.2 Selection of material	Green, green-brown shoots 3 y.o.	3 y.o. shoots	Green parts
3.3 Sample cleaning	Adhered material removal (dead and attached litter)	Adhered material removal (dead and attached litter)	Adhered material removal (dead and attached litter)
3.4 Drying	40ºC	40ºC	40ºC + an aliquot at 105ºC (d.w.)
3.5 Sample homogenization	-	Ground in mills	Ground in metal free mills

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Supplementary material: Ch. VII

Table 1: Results of the interspecies comparisons studies reported in the literature reviewed. Std: study; A: Barclay Estrup and Rinne, 1978; B: Rinne and Makinen, 1988; C: Ross, 1990; D, E, F, G, H: Wolterbeek et al., 1995; I: Ceburnis et al., 1997; J: Halleraker et al., 1998; K: Sucharová and Suchara, 1998; L, M: Galsomies et al., 1999; N, O, P: Ashmore et al., 2000; Q: Fernández et al., 2000; R: Reimann et al., 2001; S: Fernández et al., 2002; T, U: Galsomiès et al., 2003; Spp.: species compared; Ps-Hs: *Pleurozium schreberi* vs *Hylocomium splendens*; Ps-Br: *P. schreberi* vs *Brachythecium rutabulum*; Ps-Hc: *P. schreberi* vs *Hypnum cupressiforme*; Ps-Pp: *P. schreberi* vs *Pseudoscleropodium purum*; Hs-Sp: *H. splendens* vs *Sphagnum* spp; Hc-Pp: *H. cupressiforme* vs *P. purum*; Ps-Rs: *P. schreberi* vs *Rhytidialdelphus squarrosus*; n (p.s.): number of paired samples for comparison; y.o.: years old; No Corr.: no significant correlation; Sig. Correlations: significant correlations; Spp. ratio = 1: species ratio equal to 1; Spp. ratio ≠ 1: species ratio significantly different to from 1. Species that presented higher concentrations for most of the elements are highlighted in bold.

Std	Spp	n (p. s.)	Part analyzed	No Corr.	Sig. Correlations	Spp ratio = 1	Spp ratio ≠ 1
A	**Ps**-Hs	12	-	-	Pb/Zn	Pb/Zn	-
B	**Ps**-Hs	26	whole plant	-	-	Ca/Mg/Mn/ Fe/Pb	Cu/Zn
C	Ps-**Hs**	8	green parts-3 y.o. shoots	-	-	Cd/Cr/Mn/Zn	Cu/Fe/Pb/Ni/V
D	Ps-**Br**	19	green-greenbrown parts	Ti/V	As/Br/Ce/Co/Cr/Cs/Fe/ La/Mn/Na/Pb/Rb/Sb/Sc/ Se/Sm/Th/Zn	As/Br/Ce/Cr/ Cs/Fe/La/Pb/ Sb/Sc/Sm/Th	Co/Mn/Na/Rb/Sb/ Se/V/Zn
E	Ps-**Hc**	25	green-greenbrown parts	-	Cd/Cr/Cu/Fe/Ni/Pb/ Ti/V/Zn	Cd/Cr/Cu/Fe/ Ni/Pb/Ti/V	Zn
F	Ps-**Hs**	11	green-greenbrown parts-3 y.o. shoots	Cd/Cr/Ti/V	Cu/Fe/Ni/Pb/Zn	Cu/Fe/Ni/Pb/ Zn	-
G	**Ps**-Pp	49	green-greenbrown parts	Cd/Ni/V	Cr/Cu/Fe/Pb/Ti/Zn	Cd/Cr/Cu/ Fe/Ti	Pb/V/Zn
H	**Ps**-Hc	45	green-greenbrown parts	Co/Zn	V	-	Co/V/Zn

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I	**Hs**-Sp	22	3 y.o. shoots-green parts	Cd/Cu/Fe/Pb	Cr/Ni/V	-	-
J	Ps-**Hs**	25	3 most recent annual shoots	Ag/Ba/Hg/K/Sb/U	Al/As/B/Be/Bi/Ca/Cd/Co/Cr/Cu/Fe/La/Mg/Mn/Mo/Na/Ni/P/Pb/Rb/S/Sc/Se/Si/Sr/Th/Tl/U/V/Y/Zn	B/Be/Bi/La/Mn/Na/Ni/P/S/Sc/Se/Si/Sr/Th/U/Y/ Zn	Ag/Al/As/Ba/Ca/Cd/Co/Cr/Cu/Fe/Hg/K/Mg/Mo/Pb/Rb/Sb/Tl/U/V
K	Ps-**Hc**	-	2 y.o. apical parts	Al/As/Cd/Co/Cu/Fe/Mo/Ni/S/V/Zn	Cr/Pb	As/Cd/Cr/Cu/Fe/Ni/V	Al/Co/Mo/Ni/Pb/Zn
L	Hc-P**p**	10	last 3 years growth (not current year)	As/Cr/Fe/V	Zn	-	-
M	Hc-**Ps**	10	last 3 years growth (not current year)	Cr/Zn	As/Fe/V	-	-
N	Ps-Hs	23 to 30	green-greenbrown parts-4 youngest shoots	-	As/Ca/Cd/Cr/Cu/K/Mg/Na/Ni/Pb/Se/V/Zn	-	-
O	Ps-Hc	11 to 17	green-greenbrown parts	Ca/Cu/As	Cd/Cr/K/Mg/Na/Ni/Pb/Se/V/Zn	-	-
P	Ps-Rs	12 to 27	green-greenbrown parts	Na/Se	As/Ca/Cd/Cr/Cu/K/Mg/Ni/Pb/V/Zn	-	-
Q	**Hc**-Pp	48	3-4 apical cm	-	-	As/Co/Cu/Hg/Zn	Al/Cr/Fe/Ni/Pb
R	Ps-**Hs**	70	-	-	Ag/Al/As/B/Ba/Be/Bi/Ca/Cd/Co/Cr/Cu/Fe/Hg/K Li/Mg/Mn/Mo/Na/Ni/P/Pb/Rb/S/Sb/Sc/Se/Si/Sn/Sr/Th/Tl/U/V/Y/Zn/Zr	-	-

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S	**Hc**-Pp	-	3-4 apical cm	-	Al/As/Cr/Cu/Fe/Hg/Ni/Pb/Zn	-	-
T	Hc-**Pp**	10	last 3 years growth (not current year)	-	Cl/Mn/Pb/Rb/Sb/Zn	-	-
U	**Ps**-Hc	10	last 3 years growth (not current year)	-	Al/As/Co/Cs/Fe/Mn/La/Rb/Sc/Sm/V	-	-

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Table 2. Reviewed papers concerning the effect of stemflow and throughfall on moss concentrations. Std: study; A: Clement and Wittig (1987); B: Thöni and Hertz (1992); C: Rasmussen et al. (1980); D: Schilling and Lehman (2002); E: Fernández and Carballeira (2002); F: Čeburnis and Steinnes (2000); G: new analysis of data set from Fernández and Carballeira (2002). S: Stemflow effect on concentrations; T: Throughfall effect on concentrations; Fs: *Fagus sylvatica*; Pa: *Picea abies*; h.: hardwood canopies; Ppin: *Pinus pinaster*; Qr: *Quercus robur*; Jc: *Juniperus comunis*; Mh: *Mnium hornum*; Hc: *Hypnum cupressiforme*; Td: *Thuidium delicatulum*; Pp: *Pseudoscleropodium purum*; Hs: *Hylocomium splendens*; Ps: *Pleurozium schreberi*; Ea: *Eurynchium angustirete*; n.p.s.: non paired samples; p.s.: paired samples; n: number of samples (assuming balanced designs).

Std	Aim	Vegetation	Moss sp	Test/design/n	Concentration increased	Concentration decreased	No effect
A	S	Fs	Mh	-/p.s./14	Pb	-	Cd/Zn
B	S	Pa/Fs	Hc	-/p.s./2	Cd/Cu/Pb/Zn	-	-
B	S	Fs	Hc	-/p.s./18	Pb	-	Cd/Cu/Zn
C	S	Fs	Hc	ANOVA/p.s./10	Cr/Cu/Fe/Ni/Pb/V	Ca/K/Mg/Mn/Na	Cd/Zn
B	T	Pa/Fs	Hc	-/p.s./2	Cd/Cu/Pb/Zn	-	-
C	S	Fs	Hc	ANOVA/p.s./10	-	-	Ca/Cd/Cr/Cu/Fe/K/Mg/Mn/Na/Ni/Pb/V/Zn
D	T	Pinus spp./hc	Td	ANOVA/p.s./5	-	-	Pb/Cu/Cr/Ni
E	T	Ppin/Qr	Pp/Hc	ANOVA/n.p.s./22	K	-	Al/As/Ca/Cd/Co/Cr/Cu/Fe/Hg/Mg/Mn/Na/Ni/Pb/Se/Zn
E	T	Ppin/Qr	Pp/Hc	ANOVA/p.s./11	-	-	Al/As/Ca/Cd/Co/Cr/Cu/Fe/Hg/K/Mg/Mn/Na/Ni/Pb/Se/Zn
F	T	Pa/Jc	Hs/Ps/Ea	n.d./p.s./10	Cu/Ni/Zn/Cr/Fe/V	Mn	Pb/Cd
G	T	Ppin/Qr	Hc	Wilcoxon/p.s./11	-	Fe/Mg	Al/As/Ca/Cd/Co/Cr/Cu/Hg/K/Mn/Na/Ni/Pb/Se/Zn
G	T	Ppin/Qr	Pp	Wilcoxon/p.s./12	-	Mn	Al/As/Ca/Cd/Co/Cr/Cu/Fe/Hg/K/Mg/Na/Ni/Pb/Se/Zn

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Table 3: Results of the studies that assessed the effect of altitude in the concentrations of elements in moss. Std: Study; A: Groet, 1976; B: Zechmeister, 1995; C: Soltés, 199); D: Gerdol et al., 2002; E: Schilling and Lehman, 2002; F: Suchara and Sucharová, 2004; G: Sucharová and Suchara, 2004; H: Coscun et al., 2005; I: Lee et al., 2005; J: Gerdol and Bragazza, 2006; K: Pesch and Schröder, 2006. Spp.: species; Lg: *Leucobryum glaucum*; Sg: *Sphagnum girgensohnii*; Hs: *Hylocomium splendens*; Ps: *Pleurozium schreberi*; Td: *Thuidium delicatulum*; Pp: *Pseudoscleropodium purum*; Hc: *Hypnum cupressiforme*; Hp: *Hypnum plumaeforme*; y.o.: years old; a.s.l.: above the sea level; max.: maximum; Tendency: elements that showed a determined pattern despite correlations were not significant (bold: elements increased with altitude; No Corr.: no correlation; Signif. Corr.: significant correlation; positive (bold), negative (italics).

Std	Spp	Part analysed	Altitude	Tendency	No Corr.	Signif. Corr.
A	Lg	-	-	**Cd/Zn**	-	-
B	Hs/Ps	3 y.o. tissues	350 to 2260 m	**As/Cd/Co/Cr/Cu/Fe/Hg/Ni/Zn**	As/Cd/Co/Cr/Cu/Fe/Hg/Ni/Zn	**Pb/V**
C	Sg	-	750 to 2060 m	**Cd/Zn**	Cr/Cu/Fe/Mn/Mo	**Pb**
D	Hs	current year segment	1005 to 1880 m	**K**	Cd/Cu/K/Mg/Na/Zn	*Al/Ca/Co/Cr/Fe/Mo/Ni/Pb/V*
E	Td	1.5 apical cm	610-1216 m	-	Cr/Cu/Ni	**Pb**
F	Ps/Pp	2-2.5 y.o. tissues	160-930 m	-	Ba/Cs/Mn/Nd/Rb/S/Sb/Sc/Sr/Tl	*Ag/Al/As/Be/Bi/Cd/Ce/Co/Cr/Cu/Fe/Ga/Hg/In/La/Li/Mo/Ni/Pb/Pr/Se/Th/U/V/Y/Zn*
G	Ps/Pp	2-2.5 y.o. tissues	160-930 m	-	Ba/Bi/Cd/In/Mn/Mo/Pb/Sc/Se/Sn/Zn	*Al/As/Be/Ce/Co/Cr/Cu/Fe/Ga/Hg/La/Li/Ni/Pr/S/Sr/Th/U/V/Y*/**Ag/Cs/Rb/Tl**
H	Hc	green-yellowish green parts	max. = 520 m	-	As/Cd/Cu/Ni/Pb/Sb/W/Zn	-
I	Hp	1-3 apical cm	300 to 1900 m	-	Al/Ca/Co/Cr/Cu/Fe/Mg/Mn/Ni/V	*Cd/Pb/Zn*
J	Hs	current year segment	1000-2200 m	-	Al/Ca/Co/Fe	**Cd**/*K/Mg/N*/**Pb/Zn**
K	mainly Ps/Pp/Hc	-	-	-	Sb/V/Ti	As/Cd/Cr/Cu/Fe/Ni/Pb/Zn

Table 4: Reviewed papers concerning the effect of temporal variability on moss concentrations. Std: Study; A: Berg and Steinnes, 1997; B: Fernández and Carballeira, 2002; C: Zechmeister et al., 2003; D: Thöni et al., 1996; E: Engelke, 1984 (in Markert and Weckert, 1989); F: Leblond et al., 2004; G: Real et al., 2008; H: Markert and Weckert, 1993; I: Boquete et al., 2011; J: Couto et al., 2003. Spp: Species (Hs: *Hylocomium splendens*; Pp: *Pseudoscleropodium purum*; Aa: *Abietinella abietina*; Ps: *Pleurozium schreberi*; Hc: *Hypnum cupressiforme*; Dh: *Dicranella heteromalla*, acrocarpous species not usually used in biomonitoring studies; Pf: *Politrichum formosum*, acrocarpous species not usually used in biomonitoring studies). SS (n): number of sampling sites. Data from ‡Markert and Weckert (1989a) and Markert and Weckert (1989b) are included in †Markert and Weckert (1993).

Std	Sp.	SS (n)	Sampling date	Elements	Result	Trend
A	Hs	3	3 dates (June, July and September).	Li/Be/Mg/Ca/V/Cr/Mn/Fe/Co/Ni/Cu/Zn/Ga/Ge/As/Se/Rb/Sr/Y/Zr/Nb/Mo/Cd/Sn/Sb/Cs/Ba/La/Ce/Pr/Nd/Sm/Eu/Gd/Tb/Dy/Ho/Er/Tm/Yb/Lu/Hf/Ta/W/Tl/Pb/Th/U	No significant differences in concentrations ($p<0.05$) between the three sampling times.	-
B	Pp	4	12 dates (monthly)	Al/Ca/Cd/Co/Cr/Cu/Fe/K/Mg/Mn/Na/Ni/Pb/Zn	No significant differences in concentrations ($p<0.05$) between samping dates, for Cd/Hg/K/Ni/ and Pb. Significant differences for As/Cu and Zn at some sites.	-
C	Aa	1	4 dates (monthly; between July and October)	Al/Pb/V/S/Zn/Fe/Cu/Cr/Ni/Co/Mo/As/Hg	No significant differences in concentrations of the elements studied between sampling periods. Considering all elements together, a significant difference in concentrations ($p<0.001$) was observed between the various periods.	Maximum values in August
D	Hs/ Ps/ Hc	1	4 dates (once in autumn and sampling every two months between March and August)	Ag/Al/As/Cd/Ce/Co/Cr/Cu/Fe/Ga/Ge/La/Li/Mo/Nb/Ni/Pb/Th/Ti/U/V/W/Y/Zn	No seasonal differences	-

E	Dh	1	-	Cd/Pb/Zn	No seasonal differences.	-
F	Pp	1	12 dates (monthly)	Al/Ba/Ca/Fe/Hg/Li/Mn/Na/P/Pb/Si/V/Zn	Significant seasonal variations ($p<0.01$) in concentrations of Al/Ba/Ca/Fe/Hg/Li/Mn/Na/Si/V in the apical part of the gametophyte. Significant seasonal variations ($p<0.001$) in the concentrations of Na/Ca/Mn in the basal parts.	Maximum values in summer and minimum in winter (except for Na, for which the opposite pattern was observed).
G	Pp	6	13-64 dates (monthly or every two months for 1-10 years)	Hg	No seasonal differences.	-
H	Pf	1	approx. 312 dates (weekly or every two weeks for 6 years)	K/Cd/Cr/Fe/Ni/Pb/Zn/Cu/Ca/Mg/Sr/ Ba	High temporal variability (i.e. ~ 80% for Al/Cr/Fe/Ni/Pb and Ti)/ small (i.e. ~ 50% for Ba/Ca/Cd/Cu and Sr) or slight fluctuations (i.e. ~ 30% for Mg and Zn).	Maximum values in winter (January to March) and minimum in summer (July to September). The opposite pattern was observed for $Zn^{\ddagger}$ and $K^{\dagger}$.
I	Pp	2	101 dates (3-4 days during one year)	Al/Cd/Cu/Cr/Fe/Hg/K/Na/Ni/Pb/V/ Zn	Temporal variability for Na	Maximum values in winter and minimum in summer.
J	Pp	5	45 dates (weekly during 3 and a half years)	Al/K/Ca/Mg/Cu/Mn/Fe/Na/Hg/Zn	Cyclical pattern for Cu/K/Mg and Na (almost for Mn and Al).	Maximum values in winter and minimum in summer.

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GENERAL DISCUSSION

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General Discussion

Terrestrial mosses were first used to monitor the atmospheric deposition of heavy metals (at the end of the 1960s) on the basis of the observed relationship between the concentrations of these elements in moss tissues and the concentrations in the environment (Rühling and Tyler, 1968, 1970). Since then, and despite the lack of any supporting evidence, it has been assumed that mosses provide a time-integrated measure of the atmospheric deposition of heavy metals (Rühling, 1994; Čeburnis and Valiulis, 1999; Blagnyte and Paliulis, 2010; Schröder et al., 2013), with the only sources of variability in the concentrations of these elements in moss being due to the variations in atmospheric inputs and in moss growth rates (leading to a dilution effect due to the biomass increment). Some researchers have even tried to calibrate the concentrations of heavy metals in moss tissues with the concentrations in bulk deposition, in order to obtain absolute values of atmospheric deposition from moss data (Ross, 1990; Berg et al., 1995; Thöni et al., 1996; Berg and Steinnes, 1997; Schintu et al, 2005; Fowler et al., 2006). However, more recently, it has been demonstrated that mosses gain and lose elements (via inputs and outputs) (Couto et al., 2004) because as living organisms they are in very close contact and interact with the surrounding environment.

The equilibrium between moss and the surrounding environment (i.e. between inputs and outputs of elements) is determined by several factors. Thus, the way that the elements reach the moss tissues (attached to particles, ions or as gases) is important because the physicochemical characteristics of the pollutants in the deposited material determine processes such as solubilisation and leaching of elements, cation competition and anion complexation (Gjengedal and Steinnes, 1990). The element uptake capacity and other biological processes such as the rate and type of growth (Castello, 2007 and Holy et al., 2009), physiological activity (Fernández et al., 2010), phenotypic adaptations, and chemical structure (González and Pokrovsky, 2014) are also important. Finally, the equilibrium will also be affected by

environmental variables (e.g. temperature, amount of precipitation and humidity). The former variables will be conditioned by the latter (environmental) variables, which may vary considerably spatially and temporally at a small scale, and therefore any effects observed cannot be extrapolated from one time to another, or from one location to another.

According to the above, the final concentrations of heavy metals reached in moss tissues will be the result of the different and complex combinations of all these factors (not only the atmospheric inputs and moss growth), with the additional complication of large spatial and temporal variations. Thus, mosses do not integrate the pollutants that they receive, as demonstrated by the large variations in the concentrations of elements in moss tissues within periods of only a few days (Chapter I). Furthermore, the lack of a positive relationship between the time of exposure and the concentrations of elements in mosses (Chapter II) also demonstrates the non-integrative character of these organisms as biomonitors. This also indicates that the relationship between the concentrations of pollutants in mosses and in bulk deposition will not be the linear dose-response relationship described by Markert et al. (2003) as ideal for organisms used as biomonitors. In other words, the concentrations of elements in mosses do not always strictly reflect the concentrations in bulk deposition (e.g. the case of Mn described in Chapter V). This has already been pointed out in the critical review presented in Chapter IV of this thesis and further demonstrated by the general lack of correlations between the concentrations of heavy metals in mosses and in bulk deposition (Chapter VI).

In accordance with the above findings, we can state that the absolute values of concentrations of elements in moss tissues are subject to a high degree of uncertainty, and therefore mosses cannot be used for quantitative monitoring of atmospheric deposition. However, comparison of data obtained by use of the moss technique in unpolluted and polluted areas (i.e. the surrounding of industrial facilities), enables qualitative differentiation of such sites (i.e. the moss samples from polluted sites studied in Chapters I, III, V and VI always displayed higher levels of heavy metals than the unpolluted

sites). Therefore, the technique constitutes a useful biomonitoring tool for the qualitative evaluation of air quality. Nevertheless, it must be used with caution in the surroundings of industrial facilities, where indigenous mosses may have been subjected to high levels of pollution for a long time (and may have undergone phenotypic adaptations in response to toxic pollutants, as considered in Chapter III). In this type of situation, active biomonitoring will be the only reliable means of monitoring air pollutants (together with classical methods).

Obtaining reliable, comparable and useful information on air quality (even though only distinguishing polluted from unpolluted areas) requires the use of a harmonized protocol in which all the steps involved in the pre-sampling, sampling and post-sampling processes are supported by or based on scientific criteria (which is not the case in the current protocol published by the ICP Vegetation). The literature review carried out in this thesis (Chapter VII) has served to propose such a protocol covering most of these aspects. The remaining aspects, which have not been studied in detail, must be addressed in the near future.

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GENERAL CONCLUSION

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General Conclusion

Public awareness of environmental concerns has led to the development of new environmental tools to monitor air quality, such as the "moss biomonitoring technique". This has emerged as a very valuable tool as it constitutes a direct approach that enables heavy metals to be measured directly in the target organisms and also conclusions to be reached about the degree of exposure of moss to these hazardous substances and the spatial and temporal patterns of the pollutants received. It also overcomes certain limitations associated with classical measurement methods - such as the low concentrations of pollutants found during chemical analysis and the high cost of sampling equipment. Although the technique cannot substitute classical measurement methods (as it provides relative rather than absolute data on atmospheric deposition), it is the ideal complement to these methods. However, despite the fact that the technique was developed more than 50 years ago and is of recognised scientific value, it has not yet been applied in environmental policies. This has sometimes been justified by the scepticism of some people (including policy makers) who still consider the results of chemical and physical analyses to be more precise and hence, more reliable. However, at the end of this thesis, I propose that this is not the key issue.

The main problem associated with the passive biomonitoring of air quality by use of terrestrial mosses is that scientists have often tried to obtain more information than the technique is actually able to provide. Despite the well known problems associated with the factors that determine the final concentrations of elements in moss tissues (discussed extensively throughout this thesis), many researchers continue to state that the concentrations of elements in a portion of moss shoot represent the average atmospheric deposition during a certain period of time, or that the calibration of moss concentrations with those in bulk deposition will enable estimation of absolute deposition values from moss data. The low confidence in the moss biomonitoring technique is therefore probably due to the fact that many

researchers do not recognise the limitations of the technique and have not investigated either whether the limitations can be overcome or whether the results obtained in this type of study should be reinterpreted.

The results presented in this thesis, together with previously reported findings, have led us to conclude that passive biomonitoring studies with terrestrial mosses provide qualitative and/or semi-quantitative rather than quantitative information. Interpretation of the results of this type of study must therefore be reconsidered; however, this does not mean that the information obtained is not useful (as demonstrated in the different chapters of the present thesis). Such reconsideration, together with true harmonization of the technique (by publication of a protocol exclusively based on scientific criteria), should bridge the gap between the scientific application of the technique and its application in environmental policy making.

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SUMMARY

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SUMMARY (Resumen)

1 Introducción

La emisión de metales pesados a la atmósfera constituye una de las cuestiones ambientales clave en las sociedades pertenecientes a países industrializados. Todos los metales pueden llegar a ser tóxicos, sin embargo, el grado de toxicidad dependerá de cada elemento, así como de cada organismo. Algunos metales son esenciales para los seres vivos (p.e. Cr, Cu, Fe, Mn, etc.), sin embargo a elevadas concentraciones pueden causar daños (toxicidad). Otros, en cambio, resultan tóxicos incluso a pequeñas concentraciones (p.e Cd y Pb). Una vez liberados, los metales pesados tenderán a depositarse en la superficie terrestre (vía deposición seca o húmeda), en donde podrán interactuar con los ecosistemas y todos sus componentes. Es por esto que la emisión de dichos elementos a la atmósfera constituye un grave problema ambiental y de salud.

Los efectos negativos de la emisión de metales pesados a la atmósfera han generado la necesidad de medir los niveles de estos contaminantes en la misma de forma rutinaria (monitorización). Esta información es de vital importancia ya que permite controlar los niveles de estos elementos en el aire, y desarrollar políticas de regulación para proteger la integridad del medioambiente. La monitorización tradicional de los contaminantes atmosféricos constituye una tarea compleja y costosa, ya que se basa en la utilización de métodos físico-químicos para la determinación de las concentraciones de metales en la precipitación, partículas y/o gases. Sin embargo, en los últimos años se han desarrollado otras aproximaciones para la monitorización de la calidad del aire que, a pesar de no proporcionar el mismo tipo de información (valores absolutos de la deposición atmosférica), presentan ciertas ventajas.

Una de dichas aproximaciones es conocida como biomonitorización pasiva de la calidad del aire mediante musgos terrestres. Ésta consiste en la determinación de las concentraciones de metales pesados en los tejidos de estos

organismos, recogidos en el propio medio en el que se desarrollan. La técnica del musgo, como se conoce habitualmente, se basa en que estos organismos, estructuralmente muy simples y con una amplia distribución geográfica (al menos las especies empleadas en biomonitorización), obtienen la mayor parte de los nutrientes que necesitan de la atmósfera. Sin embargo, junto con los nutrientes les llegan además los contaminantes (p.e. metales pesados), que también pueden ser captados y retenidos. De acuerdo con esto, la base de la técnica es que los cambios en las concentraciones de metales pesados en la atmósfera se verán reflejados en las concentraciones de estos elementos en el musgo. Sin embargo, actualmente se sabe que esta metodología presenta ciertas limitaciones, y la mayor parte de ellas no han sido suficientemente estudiadas.

En primer lugar, algunos investigadores han asumido que los musgos integran los elementos que reciben. De este modo, las concentraciones de contaminantes encontradas en una porción determinada de los ápices de musgo, serían representativas de la deposición atmosférica media de estos elementos durante un determinado periodo de tiempo (el tiempo que los tejidos han estado expuestos). Así, los únicos factores que determinarían las concentraciones en el musgo serían la entrada de contaminantes desde la atmósfera y su crecimiento. Sin embargo, existen evidencias científicas de que los musgos sufren ganancias y pérdidas de elementos, aunque los procesos por los cuales se producen no se han estudiado a fondo. Además, el efecto del crecimiento del musgo (generación de nuevos tejidos), en sus concentraciones finales de contaminantes aún no está muy claro.

En segundo lugar, teóricamente hablando, un buen biomonitor sería aquel organismo capaz de mantener una relación lineal entre las concentraciones de contaminantes en sus tejidos y en el medio, al menos para el rango de concentraciones de trabajo. De acuerdo con esta base teórica, algunos investigadores han intentado transformar las concentraciones de elementos en el musgo en valores de deposición atmosférica, mediante la calibración de las concentraciones en el musgo con las mismas en la deposición completa. Sin embargo, hasta ahora no se ha estudiado en profundidad la relación exis-

tente entre las concentraciones en el musgo y en la deposición completa, ni tampoco los factores que podrían afectar a dicha relación.

Finalmente, algunos autores han puesto de manifiesto la posibilidad de que los musgos que han crecido durante varias generaciones en ambientes con una elevada deposición atmosférica de metales pesados, hayan sufrido adaptaciones a dichas condiciones adversas. Estos mecanismos de adaptación, permitirían al musgo a actuar como excluyente de estos contaminantes impidiendo su toxicidad y, por lo tanto, su posible efecto negativo en la "fitness" (eficacia biológica del organismo). A pesar de las importantes implicaciones de este hecho en los estudios de biomonitorización pasiva con musgos terrestres, no se ha publicado ningún trabajo en el se haya intentado confirmar o rechazar esta hipótesis.

2 Objetivos principales

Teniendo en cuenta todas estas observaciones, los objetivos principales de esta Tesis Doctoral son, en primer lugar, evaluar la fiabilidad de la técnica del musgo para monitorizar la deposición atmosférica de metales pesados. Para ello se estudiará a fondo la relación existente entre las concentraciones de estos elementos en los tejidos del musgo y en la deposición completa. Además, los factores que podrían afectar a las concentraciones en el musgo, y por lo tanto, que podrían alterar dicha relación, serán evaluados y discutidos en base a datos experimentales y a la bibliografía. Además, se estudiará el posible desarrollo de adaptaciones en el musgo que ha crecido expuesto, de manera continuada, a una elevada deposición atmosférica de metales pesados. Con toda esta información se evaluarán las limitaciones de la técnica del musgo y se propondrán posibles alternativas/soluciones en cuanto a la interpretación de los resultados de los estudios de biomonitorización pasiva con musgos terrestres.

Un segundo objetivo de esta Tesis Doctoral es la propuesta de un protocolo armonizado y basado en criterios científicos, que garantice en la medida de lo posible la calidad y comparabilidad de los resultados de los estudios de biomonitorización pasiva con musgos terrestres. Para ello se realizará una

revisión bibliográfica de todos los artículos disponibles publicados hasta la actualidad.

3 Objetivos, resultados y conclusiones específicos

3.1 Análisis de la variabilidad temporal en las concentraciones de algunos elementos en el musgo terrestre *Pseudoscleropodium purum*

El objetivo de este trabajo es evaluar la representatividad temporal de las concentraciones de elementos traza en el musgo *P. purum*, y las implicaciones que tendría la variabilidad temporal de dichas concentraciones en los resultados de los estudios de biomonitorización pasiva con estos organismos. Para esto se determinaron las concentraciones de Al, Cd, Cr, Cu, Fe, Hg, K, Na, Ni, Pb, V y Zn en el musgo recogido en dos estaciones de muestreo con distintos niveles de deposición atmosférica de metales pesados. Las muestras se recogieron dos veces por semana durante un periodo máximo de 12 meses. Los resultados de este trabajo han demostrado que las concentraciones en el musgo, tanto de contaminantes como de nutrientes, varían de forma importante en cuestión de unos pocos días. Además, se muestra cómo el error asociado a la variabilidad temporal de las concentraciones es muy elevado, más que el asociado al muestreo y análisis químico de las muestras juntos. Así, se ha podido concluir que la representatividad temporal de los resultados obtenidos en estudios de biomonitorización pasiva es muy baja. Dado que dicha falta de representatividad es prácticamente imposible de evitar (sobre todo cuando se trata de redes de muestreo a escala regional o nacional, ya que se necesitan meses para recoger todas las muestras), se recomienda el cambio de la interpretación de dichos resultados. De este modo, los resultados de los estudios de biomonitorización pasiva con musgos deberían interpretarse de forma cualitativa/semicuantitativa (ya que permiten diferenciar entre entornos contaminados y no contaminados), y no de forma cuantitativa.

3.2 Efecto de la edad en la concentración de metales pesados en segmentos de *Pseudoscleropodium purum* y en la biomonitorización de la deposición atmosférica de metales

Con este trabajo de pretende estudiar la tasa de crecimiento de *P. purum*, y evaluar su efecto (envejecimiento de los tejidos) en las concentraciones de diversos elementos en el musgo, así como sus implicaciones en los resultados de los estudios de biomonitorización. Para ello, se midió el crecimiento en diferentes estaciones de muestreo durante varios periodos de tiempo bien definidos. Además, se determinaron las concentraciones de Cd, Cu, Hg, Pb y Zn en el segmento correspondiente al tejido crecido en cada uno de los periodos. Los resultados de este trabajo han mostrado, en primer lugar, que las concentraciones de dichos elementos en los tejidos del musgo no dependen de la edad, al contrario de lo que se había expuesto anteriormente. Además, no se ha observado una relación positiva entre el tiempo de exposición y las concentraciones, esto es, el musgo no integra los contaminantes que recibe. En segundo lugar, se ha visto que la tasa de crecimiento de *P. purum* presenta una gran variabilidad, tanto espacial como temporal incluso dentro de la misma región. Por lo tanto, no es posible estimar el tiempo durante el cual una determinada porción del musgo ha estado expuesta a la deposición atmosférica de contaminantes. Por todo esto, se puede concluir que los resultados de los estudios de biomonitorización realizados en distintas regiones, en los que se ha empleado la misma especie de musgo, e incluso analizado la misma porción del ápice, no serán comparables. Así, se recomienda la selección y análisis de las partes verdes del mugo (las más activas fisiológicamente hablando) con el objetivo de minimizar el efecto del envejecimiento de los tejidos en su capacidad de carga y/o retención de los contaminantes.

3.3 Evaluación de la tolerancia del musgo terrestre *Pseudoscleropodium purum* a niveles elevados de deposición atmosférica de metales pesados: un estudio de trasplantes recíprocos

En este experimento se pretende testar la posible existencia de diferencias en la capacidad de acumulación de metales pesados del musgo *P. purum* cre-

cido de forma natural en ambientes con diversos grados de contaminación atmosférica. Para ello, se han determinado las concentraciones de Cd, Cu, Hg, Pb y Zn en muestras de este musgo trasplantadas de forma recíproca entre dos estaciones de muestreo contaminadas y una no contaminada. Los resultados de este trabajo muestran como las concentraciones de contaminantes en el musgo procedente de las estaciones contaminadas y llevado a la limpia, tienden a aproximarse a los niveles del musgo nativo de ésta última. Sin embargo, las concentraciones del musgo trasplantado desde la zona limpia a las contaminadas, llegan a superar las del musgo nativo de ambas estaciones contaminadas. De este modo, se puede concluir que el musgo que ha crecido de forma continuada expuesto a elevados niveles de contaminación se ha adaptado mediante la reducción de su capacidad de acumulación teórica. Por este motivo, en zonas con una elevada deposición de metales pesados (p.e. entornos industriales), se recomienda el uso de trasplantes (biomonitorización activa) en lugar de musgo nativo (biomonitorización pasiva).

3.4 ¿Es posible estimar la deposición atmosférica de metales pesados mediante el análisis de musgos terrestres?

Con el objetivo de averiguar si los datos obtenidos mediante la técnica del musgo permiten estimar la deposición atmosférica de metales pesados, se ha realizado una revisión crítica acerca de los estudios publicados hasta la fecha en los que se ha intentado llevar a cabo dichas estimas. La información recopilada con esta revisión indica que las correlaciones entre las concentraciones de contaminantes en el musgo y en la deposición completa son significativas sólo en un 40,1% de los casos (de éstos, el coeficiente de correlación fue superior a 0,7 en sólo un 14,1%). Además, se han identificado una serie de problemas relacionados con el diseño experimental de dichos trabajos, por lo que sus resultados no se pueden considerar concluyentes. Por último, teniendo en cuenta los resultados de trabajos en los que se ha demostrado que las concentraciones en el musgo no están determinadas únicamente por la deposición atmosférica, esta revisión permite concluir que la deposición atmosférica de metales pesados no puede ser estimada de forma fiable a par-

tir de las concentraciones en el musgo.

3.5 ¿Son los musgos terrestres buenos biomonitores de la deposición atmosférica de Mn?

El Mn es un elemento que nunca ha mostrado correlaciones significativas entre sus concentraciones en el musgo y en la deposición completa. Por este motivo, se han realizado una serie de experimentos de campo y laboratorio con el objetivo de determinar si *P. purum* es un buen biomonitor de la deposición atmosférica de Mn. Los resultados de los diversos experimentos han mostrado que la carga de Mn por parte del musgo se puede ver reducida/limitada en determinadas condiciones, a pesar de que los aportes al musgo se mantengan. Sin embargo, cuando la deposición atmosférica de este elemento es elevada y, sobre todo se produce en forma particulada, las concentraciones de Mn en el musgo varían de acuerdo con su deposición atmosférica. Por este motivo, se puede concluir que el Mn debería ser excluido de las redes de biomonitorización extensiva de la calidad del aire con musgos terrestres. En cambio, la técnica del musgo podrá ser utilizada para monitorizar el Mn en condiciones muy concretas (p.e. cuando el musgo esté expuesto a elevados niveles de Mn ligado a material particulado).

3.6 Relación entre la deposición completa de elementos traza y sus concentraciones en el musgo terrestre *Pseudoscleropodium purum*

Con el objetivo de evaluar si la falta de correlación encontrada en la bibliografía entre las concentraciones de metales en el musgo y en la deposición completa se ha debido a las limitaciones en el diseño experimental de dichos trabajos, se han recogido simultáneamente muestras de *P. purum* y de deposición completa en 21 estaciones de muestreo con diversos grados de contaminación. Además, se han determinado las concentraciones de Cd, Cu, Hg, Pb y Zn en los dos tipos de muestra y la relación existente entre ambas variables ha sido estudiada. Los resultados de este trabajo muestran que, en primer lugar, el número de correlaciones significativas ha sido muy limitado y depende del modo en que se analicen los datos; en segundo lugar, que la

forma de la regresión entre ambas variables (i.e. pendiente y signo), varía en función de la combinación de réplicas musgo vs. deposición completa utilizada; en último lugar, que los factores de bioconcentración máxima varían según los elementos, la estación de muestreo y el tiempo de exposición. Por todo esto se puede concluir que la relación entre la deposición completa de metales y sus concentraciones en el musgo viene mayormente determinada por factores distintos de la propia cantidad de metal depositado. Por lo tanto, parece obvio que los musgos no permiten obtener valores absolutos de la deposición atmosférica de contaminantes. Así, sólo se podrá recomendar el empleo de la técnica del musgo cuando se vaya a realizar una interpretación cualitativa de los resultados, mientras que si el objetivo es obtener valores absolutos, se recomendará el uso de métodos físico-químicos.

3.7 Revisión crítica de los protocolos y la literatura acerca de la metodología de la técnica del musgo

A pesar de que la aplicación de la técnica del musgo para biomonitorizar la calidad del aire ha mostrado tener ciertas limitaciones, ésta es perfectamente válida para evaluar los patrones espaciales y temporales de variación de los contaminantes atmosféricos y para detectar focos de contaminación. Sin embargo, la falta de un protocolo armonizado limita considerablemente la comparabilidad y la calidad de los resultados obtenidos en este tipo de estudios. Por este motivo, el objetivo de este trabajo consiste en proponer un protocolo armonizado y basado en criterios científicos, para la aplicación de de la biomonitorización pasiva con musgos terrestres. Así, se han seleccionado un total de 461 trabajos publicados entre 1972 y 2014, de los cuales se revisaron finalmente 369 (una vez descartados aquellos a los que no se tenía acceso, y los que se desviaron de la aplicación de la técnica del musgo para biomonitorizar la deposición atmosférica de metales pesados). Los resultados de esta revisión muestran que, en líneas generales, los científicos se han preocupado más por la mera aplicación de la técnica, que por la investigación de los aspectos metodológicos que podrían limitar los resultados obtenidos con la misma. Por este motivo, se ha recopilado toda la información posible

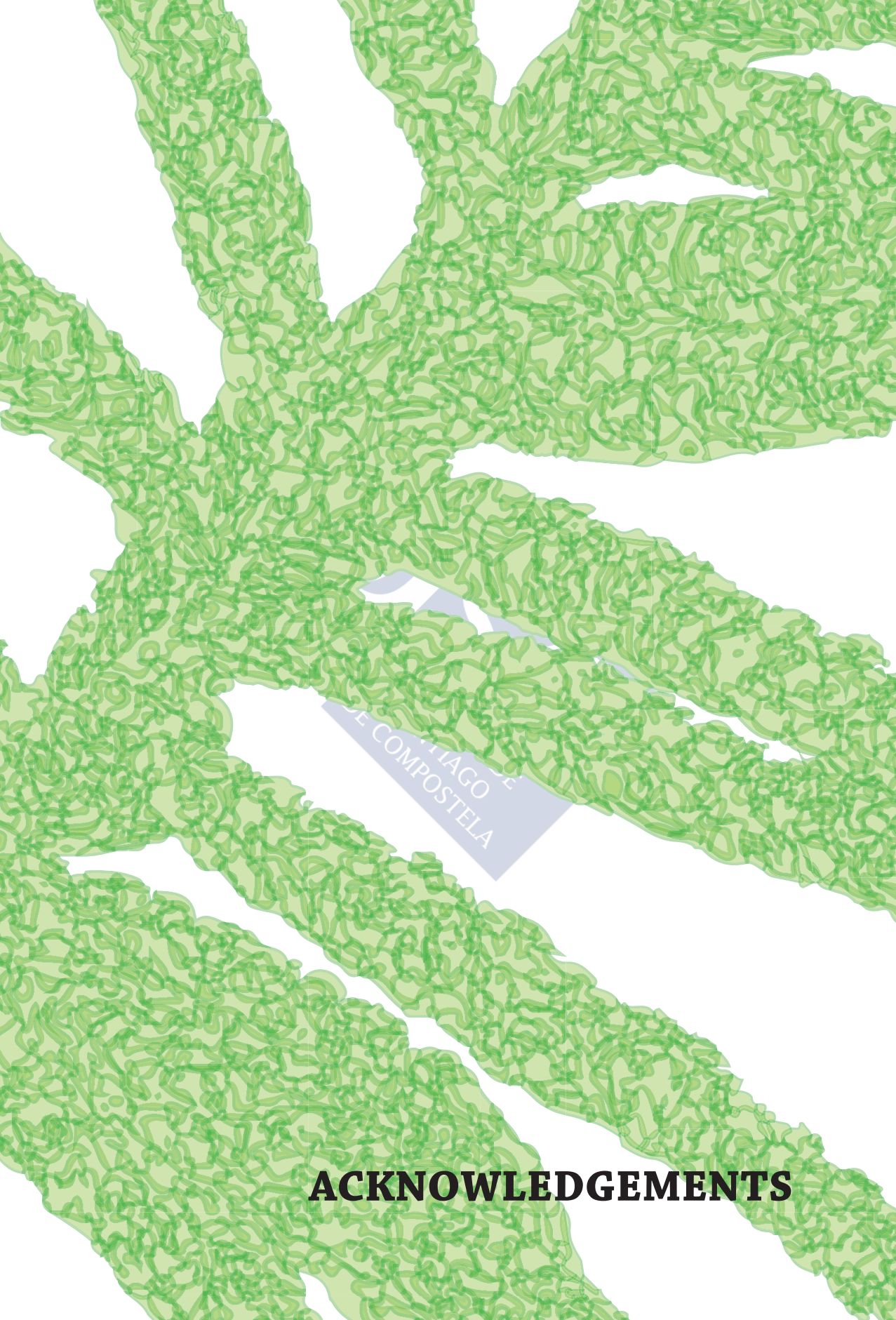
en cuanto a los trabajos metodológicos y, en base a sus resultados, se han realizado propuestas con respecto a los aspectos metodológicos involucrados en la aplicación de la técnica. Cuando no se han encontrado estudios que justifiquen la selección de algún aspecto, se ha resaltado la necesidad de investigaciones futuras.

4 Conclusión general

Los resultados de esta Tesis Doctoral han demostrado que el modo en el que se ha venido utilizando la información obtenida mediante la biomonitorización pasiva de la calidad del aire con musgos terrestres no ha sido el adecuado. Éste es el motivo por el cual las conclusiones que se han alcanzado anteriormente en muchos trabajos no son del todo fiables, y probablemente la causa de que el uso de esta técnica no se haya extendido fuera del ámbito científico (p.e. para su aplicación práctica dentro de programas o políticas ambientales). Teniendo en cuenta esto se puede concluir que el uso adecuado de la técnica del musgo, junto con una verdadera armonización de los protocolos de aplicación de la misma, probablemente implicaría un salto desde la mera aplicación científica a su uso práctico en el control de la calidad ambiental.

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Acknowledgments

Resulta difícil resumir en pocas palabras todo lo que podrías decir a la gente que te ha acompañado durante este largo viaje. Sin embargo, y como hay que hacerlo así, allá vamos...

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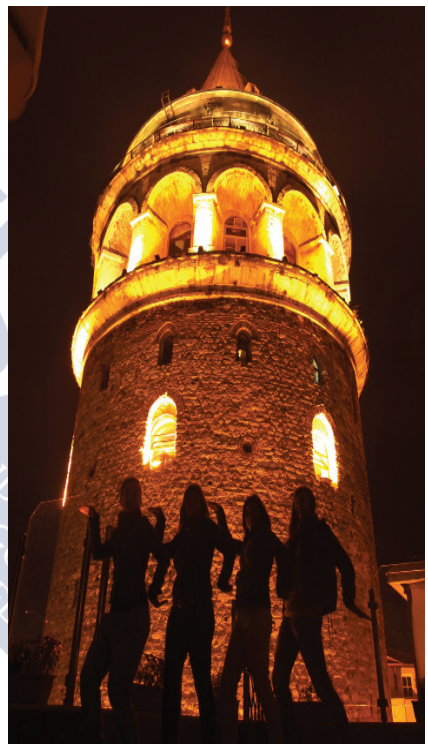














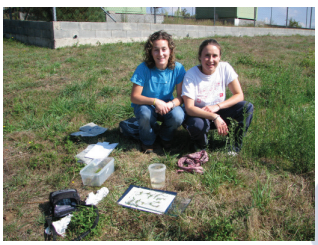
















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