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Active biomonitoring of the air quality with terrestrial mosses: standardization and optimization of the technique

Memoria que presenta
Ángela Ares Pita
para optar al Grado de Doctora en Biología

Fdo: Ángela Ares Pita
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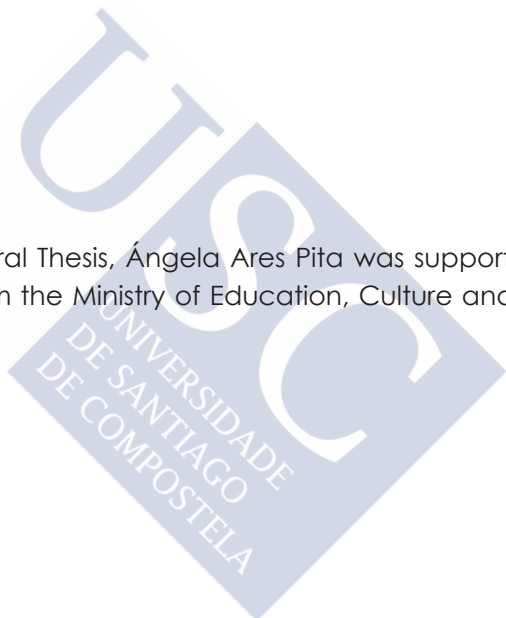
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A mi madre
y a Pablo

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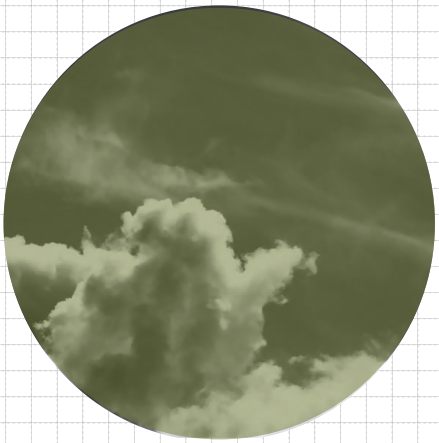
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General Introduction



1.1. Justification

1.2. State of Art

1.3. Objectives

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1.1. Justification

Atmospheric contamination is one of the most pressing problems facing the world at present. The emission of contaminants, which has been increasing ever since the Industrial Revolution, accelerated during the Second World War as a result of the technological advances made during that period. Thus, although the quality of life has improved considerably since these times, other basic aspects such as the integrity of ecosystems and human health are being seriously and perhaps even irreversibly affected. In the European Union, several directives have been promulgated (first 96/62/EC and later 2008/50/EC) with the aim of intensifying atmospheric monitoring. These directives oblige the member states to produce periodical reports on the air quality in their territory and to ensure that the European population is aware of the levels of contamination and associated risks.

However, monitoring atmospheric contaminants is a complicated task, as the concentrations and emission forms (i.e. gaseous and/or particulate) of pollutants are very variable in space and time. On the one hand, the techniques used to evaluate atmospheric levels of the main contaminants (mainly CO, SO_x, NO_x and particulate matter) are accurate and provide data within a short period, although data obtained from automatic devices are too limited in number to describe spatio-temporal trends in pollutants. On the other hand, there are important technical and economic difficulties associated with measuring other air contaminants included in the European Directives, such as some heavy metals (i.e. As, Cd, Hg, Ni and Pb) and PAHs. These compounds have serious damaging effects on human health and ecosystems, as recognised by diverse international agencies such as the United States Environmental Protection Agency (USEPA) and the Agency for Toxic Substances and Disease Registry (ATSDR). As a result

of the lack of availability of data on the concentrations of specific contaminants, implementation of corrective measures for improving environmental health is very complicated. Therefore, taking into account all of the above, there is currently a need to develop new tools, specifically bio-tools, which would enable the simple, economic and robust monitoring of air quality. Of the biomonitors available, terrestrial bryophytes (specifically mosses) are the most suitable because of their characteristics (Bargagli, 1998). Their properties confer mosses with a high cation exchange capacity and suitable morphology for fixing and accumulating contaminants that are present in gaseous or particulate form, and of both organic and inorganic nature, which are derived from atmospheric deposition.

The use of mosses has enabled the establishment of large scale networks for extensive-type studies (e.g. regional or national studies) and also the establishment of small scale networks for more intensive-type studies (e.g. in urban or industrial areas). Although native mosses have been identified as more suitable for the former type of study, moss transplants are usually used for the second type as native mosses are often not present in industrialized and urban areas. Active biomonitoring studies have been successfully carried out in cities such as Belgrade (Aničić et al., 2009a), Budapest (Naszradi et al., 2007), Naples (Giordano et al., 2005) and Oporto (Vasconcelos and Tavares, 1998), among others, and in industrial facilities such as aluminium smelters (Aboal et al., 2008), electricity power stations (Carballeira and Fernández, 2002; Couto et al., 2004), chlor-alkali plants (Lodenius, 1998; Fernández et al., 2000), and copper smelters, among others. The use of moss transplants offers other advantages compared with use of native moss (see e.g. Aničić et al., 2009a,b; Gailey and Lloyd, 1986a; Sun et al., 2009), including a reduction in the natural variability in moss, better interpretation of temporal variability (as the initial concentra-

tion and the period of exposure are known), and minimization of the edaphic influence on the moss growing naturally in an area.

Of the different types of moss transplants, the "moss bag" technique is the most common. The technique was first used by Goodman and Roberts (1971) and involves exposure of moss samples held within mesh bags in order to monitor the presence of contaminants, mainly inorganic contaminants, in the air. Despite the advantages of the use of moss bags over native moss, and the fact that both techniques were first used at almost the same time (more than 40 years ago), the number of scientific publications concerning native moss is much higher than the number concerning moss transplants (around 400 compared with 130, located through SciVerse SCOPUS). In addition, a European programme (The International Cooperative Programme on Effects of Air Pollution on Natural Vegetation and Crops [ICP-Vegetation]), in which native bryophytes are sampled every 5 years, has been created to biomonitor the atmospheric deposition of heavy metals. In the last survey, moss samples from almost 30 European countries were included (Harmens et al., 2013). In contrast, all of the available information about the "moss bag" technique is restricted to the scientific research field.

One of the main problems associated with the use of the "moss bag" technique is that the first authors to describe the technique (Goodman and Roberts, 1971) did not provide a detailed protocol for the preparation and exposure of the moss bags in the field. This led to free interpretation of the method in subsequent studies, and numerous different protocols are used nowadays. The lack of standardization of the technique limits interpretation of the results obtained and hampers comparison of the conclusions reached in different studies.

In the case of the use of native moss, although revision of the methodology is also required, the protocol first proposed by Rühling

(1989) has been applied with minimal modifications until the most recent version (Harmens et al., 2005). The European Committee for Standardization has also published a standard method for biomonitoring air quality (CEN/TC 264/WG 31: Biomonitoring methods with mosses and lichens).

The first difficulty in developing a standardized protocol is that studies addressing methodological aspects are very scarce. These aspects are related to key steps in the technique, such as the selection and preparation of moss, preparation of the bags, exposure conditions and post-exposure treatments. In the next section, the "State of the art", a critical literature review of the methodology used to date in applying the moss technique was carried out. This will reveal to exact degree to which each of the different methodological aspects have been standardized. On the basis of this information, it will be possible to propose a standard protocol that will serve as the basis for further studies aimed at improving the technique and finally applying it in the field.

Among the key steps that must be taken into account in applying the "moss bag" technique, only one has been given particular attention in the literature: the pre-exposure treatment of moss (e.g. devitalizing treatment during preparation of the moss: Giordano et al., 2009). These authors assumed that the accumulation of elements in moss is mainly due to passive processes and therefore that the use of a devitalizing treatment would modify the composition of the elements in the moss, but would not modify the moss morphology. This would ensure that particulate material would still be intercepted by moss. The convenience of replacing live moss with devitalized moss for biomonitoring purposes has been reported in several studies (e.g. Gailey and Lloyd, 1986; Castello, 1996; Adamo et al., 2007). All of these authors state that this step overcomes interference with element uptake due

to the moss metabolism and thus allows a more direct comparison between the results of different surveys. However, to date, the possible metabolic interference in the uptake of heavy metals by moss transplants has not been investigated. Chapter I describes a field experiment with irrigated transplants, aimed at clarifying whether growth of the moss interferes with or contributes to the uptake of contaminants during the exposure period.

Once determined whether devitalized moss should be used, the proposed protocol, included in the "State of the art" section, must be standardized by a detailed study of the key methodological steps in the application of the "moss bag" technique. Optimization of each of these steps should be done on the basis of data replicability and capture of a "signal" concentration that is significantly higher than that obtained in unexposed (control) moss. Thus, in Chapter II, the following methodological aspects were chosen from among the most variable in the use of the technique and that have the greatest influence on the results obtained: selection of species, the relationship between moss weight and bag surface area, duration of exposure, and height of exposure. This process represents a further step in the standardization of the "moss bag" technique, thus improving the quality and reproducibility of the data.

One of the key requisites for the biomonitoring technique to be considered as a valid alternative to traditional methods is that the concentrations of elements in the biomonitor organism should reflect atmospheric levels. In the case of passive biomonitoring with native mosses, there is some evidence of a lack of a direct relationship between the concentration of several heavy metals in moss and the concentration in the total atmospheric deposition (see Aboal et al., 2010). Published studies addressing this aspect in the "moss bag" technique are much more scarce, and in any case all studies are carried out with live moss

and the results are, therefore, not comparable. Thus, considering the conclusions reached in previous chapters, Chapter III investigates the possible correlation between the heavy metal content in mosses in standardized moss bags and the total deposition. This enables evaluation of whether the lack of a linear relationship between the concentrations of elements in the moss and in the bulk deposition found in other studies is due to metabolic interference in the uptake process.

Finally, once optimized, the technique must be validated in the field. Use of a standardized unified methodology to obtain realistic information about the emissions of pollutants is essential in modern day society.

The main advantages of the technique are that it does not require any source of energy and that it is inexpensive, thus enabling intensive surveys to be carried out and the monitoring of a large number of different contaminants, both organic and inorganic, in the same sample. Chapter IV describes the practical application of the standardized tool, to demonstrate its usefulness. Use of the "moss bag" technique will enable collection of the data required to construct maps of spatio-temporal trends in the presence of trace elements and PAHs in urban areas. The availability of such information will allow the identification of (high) risks to human health and/or ecosystems.

1.2. State of the Art

Moss bag biomonitoring: A methodological review

2012. Science of the Total Environment 432 / 143-158.

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Abstract

Although the moss bag technique has been used for active biomonitoring for the past 40 years, there is still no standardized protocol that enables application of the technique as a tool this review paper is to evaluate the degree of standardization of each of the variables that must be considered in applying the technique (i.e. the variables associated with preparation of the moss and moss bags, exposure of the bags, and post-exposure treatment). For this purpose, 112 scientific papers that report the methods used in applying the moss bag technique were consulted. Finally, on the basis of the conclusions reached, we propose a protocol that will enable each of these variables to be investigated separately, with the final aim of developing a standardized methodology.

Keywords: Moss bags, active biomonitoring, air pollution, technique standardization.

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1. Introduction

Monitoring the air for pollutants is a complex technical task, and techniques for monitoring atmospheric contamination have been the object of intensive research in past decades (Helsen, 2005). Such techniques usually involve the application of physical and chemical methods to quantify the levels of contaminants in the atmosphere. However, an alternative way of monitoring such contaminants is to use organisms, such as terrestrial mosses, as biomonitors. The use of terrestrial mosses has certain advantages over currently employed methods, including simplicity, reliability, cost effectiveness and the lack of the need for electrical energy. On the other hand, the use of mosses can help solve a fundamental problem associated with traditional methods of monitoring air pollution, i.e. the lack of specific techniques for routine measuring some pollutants (i.e. heavy metals, PAHs, radionuclides). As a result of this, several pollutants are not included in air quality monitoring in some countries. The use of mosses enables simultaneous monitoring of a large number of pollutants with the same sample. Biomonitoring with moss is also low-cost and has a very high spatial resolution that cannot be obtained with traditional techniques. Nevertheless, there are some disadvantages associated with biomonitoring, such as the fact that information is only obtained after relatively long periods, and daily or hourly measurements are not possible.

Two types of biomonitoring are clearly differentiated in the existing literature on the use of mosses to evaluate atmospheric contamination: (i) passive biomonitoring, using moss that grow naturally in a particular area, and (ii) active biomonitoring, by transplanting moss from other locations. The latter technique involves co-

llecting moss from relatively unpolluted habitats, then cleaning, selecting and pretreating the material, before exposing the samples in a different environment. The use of moss transplants resolves various problems associated with the use of native moss. First, it overcomes the scarcity or absence of moss in certain environments (i.e. industrial and urban areas). Second, it reduces the high degree of variability in the uptake of contaminants by native moss within the same sampling site (Aboal et al., 2006; Fernández et al., 2002). According to, Castello (1996) and Varela et al. (2010) the coefficients of variation (CV) for a particular element are lower between replicates of moss transplants than between subsamples of native moss at a sampling site (e.g. the CV for Pb in moss bags ranged between 14 and 21% for 10 replicates exposed for 2 months, in comparison with a CV that ranged between 33 and 93% for 50 samples of authochthonous moss collected at a sampling site). Third, transplants can be used more conveniently for the interpretation of the temporal variability in the results; while initial concentration and exposure period are known when moss bags are used, the concentrations in native moss are assumed to represent the contamination corresponding to a particular site, without taking the temporal variability into account, and a high degree of variability has been documented in short periods of time, i.e. 3-4 days (Boquete et al., 2011a). Fourth, transplants minimize the possible edaphic influence on the concentrations of certain elements (see e.g. Bargagli, 1995; Berg & Steinnes, 1997); while transplants are usually exposed at a certain height above the ground, native moss is subjected to rain splash and accumulation of soluble soil compounds during periods when there is close contact between soil and water (Berg & Steinnes, 1997). Finally, phenotypic and/or genotypic adaptation may occur in contaminated environments, thus modifying the tissue concentrations of contaminants (Fernández et al., 2000; Fernández & Carballeira, 2000; Tabors et al., 2004); this problem can be solved by transplanting moss from uncontaminated areas.

Moreover, the effect of contaminants on the physiology of native mosses is difficult to evaluate, and it is impossible to isolate their effects from those of other environmental variables. However, the use of irrigated moss transplants removes certain environmental stressors (e.g. hydric stress or stress from solar radiation), thus isolating the effect of contamination.

The "moss bag technique" is the most common type of active biomonitoring with terrestrial mosses that is reported in the literature. The technique was originally introduced by Goodman & Roberts (1971), and consists of the exposure of moss samples held within mesh bags, in order to monitor the occurrence in the air of contaminants. In the literature consulted (112 papers published between 1971 and 2011, located through SciVerse SCOPUS), the technique was mainly used to monitor levels of inorganic contaminants, mostly heavy metals (86%), and to a les-

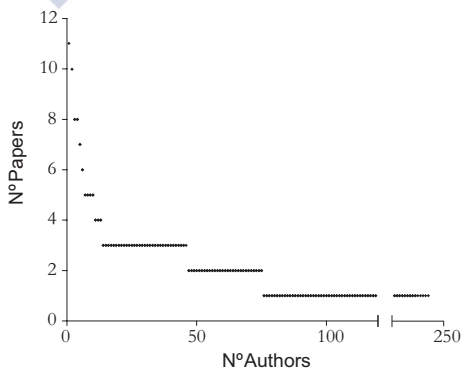


Fig. 1. Number of papers on active biomonitoring of air quality with terrestrial moss, in relationship to number of authors.

ser extent, organic contaminants such as PAHs (4%), PCBs (1%) and others (9%). Of the heavy metals studied, Pb was determined in 67 studies, followed by Zn (55), Cu (52), Cd (49), Fe (46) and Ni (43).

However, although the moss bag technique has been used for 40 years, standardized protocols have unfortunately still not been developed. Such standardized protocols would include: (i) preparation of the moss; (ii) preparation of the transplants; (iii) exposure of the transplants and (iv) post exposure treatment. The scarce importance given to standardization of the technique is reflected in the fact that some studies provide very little information and/or contradictory information (e.g. Kupiainen & Tervahattu, 2004), and sometimes even information that is difficult to access (Lodenius, 1998), about the methods used. Some authors even claim to have used "standardized" transplants, although they have not followed the recommendations given in previously published papers (see e.g. Solga et al., 2006; Temple et al., 1981). The lack of such protocols hampers comparison of the results obtained in different studies, and sometimes limits the conclusions that can be reached.

Goodman & Roberts (1971), the first authors to apply the technique, used procedures that were not based on the results of previous research. Only three years later, Little & Martin (1974) introduced modifications, as did many other researchers, so that several ways of applying the technique have been reported. These range from simple methods in which the moss tissues are barely handled (e.g. transplantation of moss mats, see e.g. Acar, 2006; Samecka-Cymerman & Kempers, 2007) to methods involving complex pretreatment and exposure systems (see e.g. Amblard-Gross et al., 2002; Couto et al., 2004a; Rivera et al., 2011). As a result, many methods have been used only once by the same group of researchers. Moreover, as some 68% of the authors have only published one paper on the topic, numerous methods have been reported (Fig. 1). Therefore, despite the advantages associated with the technique, its use has been limited to scientific research, and it has not been implemented by the responsible authorities to monitor atmospheric contamination. Moreover, use of the technique is largely limited to Europe (i.e. 80% of published papers; Fig. 2).

Despite all of the above, several authors have focused their research on methodological aspects, with the aim of establishing a standardized methodology. The first studies of this type were those carried out by Gailey & Lloyd (1986b,c,d) to investigate the relation between weight and surface area of the moss, and the optimal time of exposure in moss bags (Fig. 3). Unfortunately, their conclusions were scarcely noted in posterior studies. In the following years, very few studies addressed methodological questions, but from 2007 onwards (i.e. in 60% of the studies relating to the methodology; Fig. 3), a small number of researchers who addressed this problem have focused their research on aspects such as the treatment of the moss prior to exposure (Adamo et al., 2007, 2008a; Giordano et al., 2009; Fernández et al., 2010; Tretiach et al., 2007).

The present review considers 4 key aspects in standardizing the technique of active biomonitoring of air quality: (i) selection and preparation of the moss tissue; (ii) preparation of the transplants; (iii) exposure of the transplants, and (iv) post exposure treatment of the transplants. After reviewing and discussing the literature consulted, we indicate, for each aspect, whether the particular stage of the process can be standardized or whether further research is required.

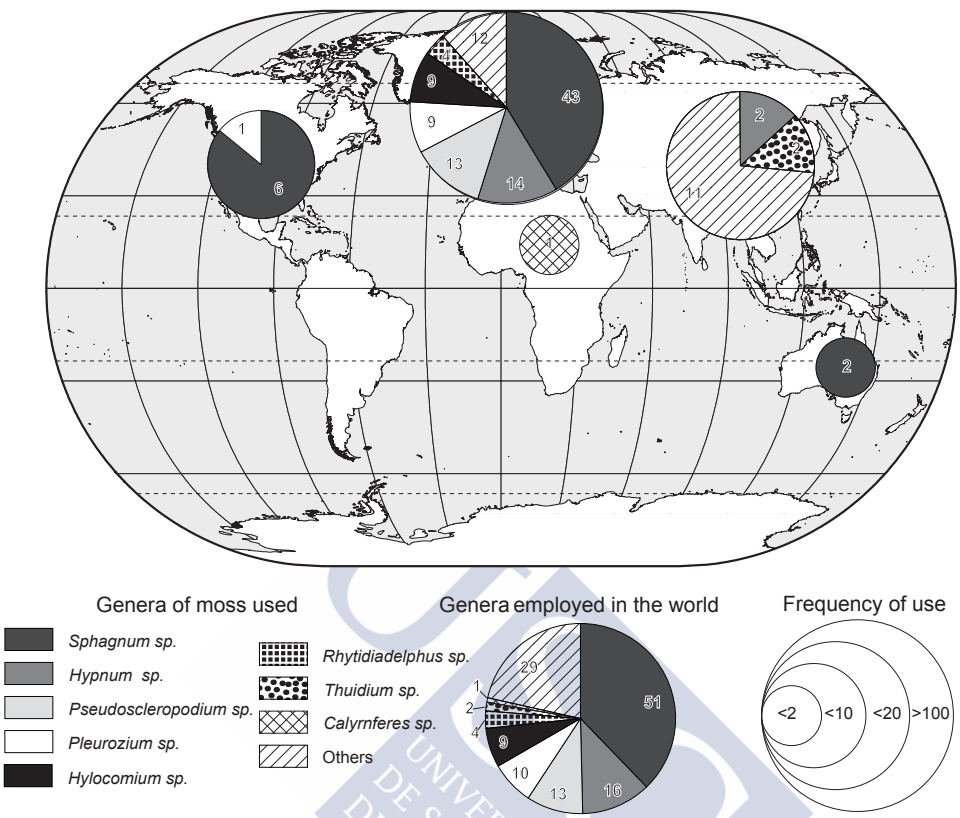


Fig. 2. Representation of the different genera of moss used in the active biomonitoring technique on different continents and throughout the world. The frequency of use of each genus in this type of study is indicated for each continent and on a global scale.

The overall aim of the present paper is to review a wide set of articles dealing with the moss bag technique in order to propose a harmonised methodology for preparing and exposing moss transplants that meets the following requisites: (i) that the transplants are easy to prepare and handle; (ii) that they enable replicable results to be obtained; (iii) that they are capable of capturing high concentrations of as many contaminants as possible, and (iv) that they are efficient at capturing contaminants, highlighting their occurrence in the air in a reasonable timespan.

2. Selection and preparation of the moss

Preparation of the moss prior to transplantation always includes selection of the species and usually selection of the portion of the shoot, as well as diverse pre-exposure treatments.

2.1. Selection of species

The particular species used will greatly affect the results (Castello, 2007; Culicov & Yurukova, 2006), and comparable results may not be obtained with different species. However, to date several different species (i.e. 45) have been used, and in

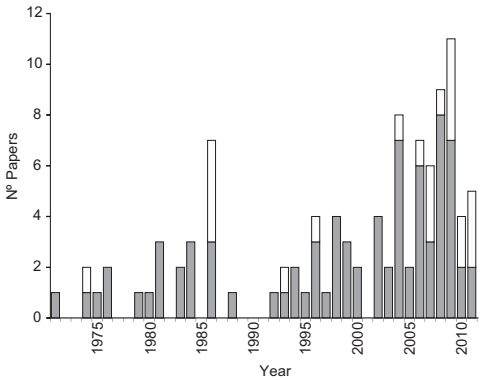


Fig. 3. Number of papers on active biomonitoring of air quality with terrestrial moss, published in different years. The columns represent the number of papers that address methodological aspects (white bars), and the number in which the technique is used to monitor contamination (grey bars).

some cases this information is omitted (Tan et al., 2000) or only the genus is indicated (see e.g.: Carpi et al., 1994; Santamaria & Martin, 1997; Wegener et al., 1992). Thus approximately 55% of the species have only been used on one occasion. Given this diversity of species, we have grouped them to show the frequency with which the different genera have been used in previous studies (Fig. 2). Moss bags have most often been prepared with species of the genus *Sphagnum*, followed by other genera widely used in Europe, represented by *Hypnum cupressiforme*, *Pseudoscleropodium purum* and less often *Pleurozium schreberi* and *Hylocomium splendens.* The main reason for selecting a particular species appears to be its presence and abundance in the study region, which has enabled in some studies the simultaneous use of native and transplanted moss (see e.g. Fernández & Carballeira, 2000; Kosior et al., 2008; Ratcliffe, 1975). Selection of the species should take into consideration the following aspects: (i) widely distributed and large-sized plants should be preferred; (ii) selected species should have some structural and physicochemical characteristics that allow efficient uptake of contaminants from the atmosphere; and (iii) the selected species should be one most commonly used and about which most information exists.

Firstly, the availability of large specimens will facilitate handling and reduce the possibility of loss of material during exposure (i.e. selection of the portion of shoot to be exposed, cleaning, etc.).

Secondly, the capacity of the different species of moss to capture contaminants depends on the following: (i) the morphology of the shoots, which determines the capacity of the moss to retain particles, as well as the circulation of gases and water around the tissues; (ii) the cation exchange capacity (CEC), which is mainly linked to the quantity of uronic acids in the cell wall and the outer parts of the cell membrane, and their degree of methylation (Carballeira et al., 2008; Richter and Dainty, 1989); and (iii) the specific surface area (i.e. $m^2\ kg^{-1}$), as obviously the area exposed to the environment will increase with the surface area and roughness of the tissues. The capacity of the moss to accumulate elements will therefore increase with the number of phyllids per caulid (Sun et al., 2009). Clough (1975) concluded that the rough surface and hairiness of moss reduce subsequent removal of elements by blow-off, bounce-off and rain wash. Adamo et al. (2007) explained the better efficiency in metal accumulation of *Hypnum cupressiforme* compared with lichen *Pseudevernia furfuracea* on the basis of the higher moss specific surface area ranging between 81 and 136 $m^2\ kg^{-1}$.

When analyzing the differences in the capacity of different species of moss to capture contaminants, studies carried out with native mosses should not be taken into account. In such studies, the capacity for accumulation depends, in addition to

the aspects mentioned above, to the form of growth (i.e. erect shoots compared with prostrate shoots, or scattered moss shoots compared with densely crowded shoots) (Castello, 2007; Holy et al., 2009), to the substrate on which they grow and on the microenvironments inhabited by the species, whereas in transplants, these factors are not relevant. This explains why on comparing pairs of species, contradictory results may be obtained depending on whether moss transplants or native moss is considered (e.g. Castello, 2007).

Very few of the studies reviewed have compared the morphological and physicochemical characteristics of different transplanted species, and the only information available in this respect refers to the final effect of these characteristics on the concentration after transplantation in the same locations in the same exposure periods. In regard to studies that compare the most commonly used types of moss, Ratcliffe (1974) concluded that the Pb concentrations in bags of *H. cupressiforme* was only 66% of that in bags of *Sphagnum sp*. This was confirmed by Culicov & Yurukova (2006) on comparing *H. cupressiforme* and *Sphagnum girgensohnii*. Furthermore, Castello (2007) exposed spherical bags of *H. cupressiforme* and *P. purum* simultaneously in an industrial environment, and concluded that the latter was a better accumulator, showing similar or higher accumulation and lower loss of almost all elements than the former. Other authors such as Čeburnis & Valiulis (1999) demonstrated that there is no significant difference between *H. splendens* and *P. schreberi* as regards quantitative uptake of metals. Tremper et al. (2004) compared *P. schreberi* and *Rhytidiadelphus squarrosus*, and found higher concentrations of contaminants in *P. schreberi*. Finally, only one study (Yurukova & Ganeva, 1997) compared two species of the genus *Sphagnum*: *Sphagnum teres* and *Sphagnum capillifolium*, and concluded that the former exhibited a higher capacity for accumulation. In all of the above-mentioned studies the moss samples were assumed to be alive when exposed.

There is an obvious need to select one species for standard use in moss bags. This would make possible and reliable the comparison between monitoring data obtained in different environments. To enable the widespread use of this species the best option would be its cultivation in the laboratory; therefore, it is crucial that the selected species can be cultivated in the laboratory producing high biomass in a relatively short time.

The possibility to obtain in the laboratory enough moss biomass to be used as a biotool in bags is a solution to a series of problems intrinsic to the use of mosses growing naturally in an area: (i) the species chosen may not always be available in a particular study area (Adamo et al., 2008a), due to population fluctuations or changes in the status of the species to be protected; (ii) problems associated with field identification (e.g. species of the genus *Sphagnum*) and taxonomic characterization (e.g. the *H. cupressiforme* complex; Smith, 1997), (iii) native moss is also highly heterogeneous, as both the physicochemical characteristics and the tissue concentrations of contaminants fluctuate over time, as well as between and within different locations. The heterogeneity of the material used implies a low level of replicability. Depending on the initial concentrations and/or the physicochemical characteristics of the moss used for transplants, the final concentrations will vary, despite being exposed to the same level of contamination.

The isolation and cultivation of a specific clone to produce homogeneous material for the moss bags would solve these

problems. A clone of aquatic moss has been isolated for use in biomonitoring the quality of continental waters (Rausch de Traubenberg & Ah-Peng, 2004), and more recently, a clone of the terrestrial moss *Ceratodon purpureus* has been isolated (Fabure et al., 2010) for use in moss bags. Unfortunately, we did not find any information about laboratory culture of any of the commonly used moss species. However, culture of *Physcomitrella patens* -by vegetative propagation of the filamentous juvenile protonema tissue in suspension culture- has been optimized, thus enabling large scale production of biomass of the species for biotechnological purposes (Decker and Reski: 2004, 2007, 2008). However, both *C. purpureus* and *P. patens* are small acrocarpic species and their morphology and size may determine structural characteristics (i.e. surface to mass ratio) that would make them unsuitable for handling and capture of contaminants.

With the available information regarding the moss species most commonly used in active biomonitoring, it appears that species of the genus *Sphagnum* best fit the criteria for the suitability of moss species in the moss bags technique. They are of a suitable size for handling and have been cloned (Såstad et al., 1998), although they have not been cultivated for use in moss bags. As regards the physicochemical properties of *Sphagnum spp.,* the leaves constitute about two thirds of the dry biomass and provide a very large surface area for cation exchange processes and capture of fine airborne particles (Bargagli, 1998), the hyalocysts have large pores that can act as a trapping system for airborne particulate (Giordano et al., 2005), and the cation exchange capacity has been estimated to be 0.9 -1.5 meq g^{-1} dry weight (i.e. higher than that of other mosses, 0.6 - 1.1 meq g^{-1})(Temple et al., 1981).

2.2. Selection of material

Once the species has been selected for use in the moss bags, the next step is to decide which portion of the shoots should be used. The results may vary depending on the material selected, because the older parts of the stems will contain different amounts of some elements than younger tissues (Tavares & Vasconcelos, 1996; Leblond et al., 2004; Fernández et al., 2010). However, this information is not provided in 56% of all the papers reviewed and in the other papers (44%) the following options were chosen: apical portions of the shoots (19.4%), green parts (10.2%) and entire shoots (14.3%).

The aim of selecting the material is to obtain material that is as homogeneous as possible for transplantation. The shoots of naturally growing moss are highly variable in size, and therefore the portion of older and green parts will also vary. As the concentrations in these portions will differ (see above), and probably also their capacity for bioconcentration the use of whole shoots should be discounted.

In spite of the different substrate and exposure to atmospheric deposition, green shoots of epiphytic and epilithic *Hypnum cupressiforme* from the same site show a rather homogenous chemical composition (CV <20%) for most major and trace elements, the main exceptions being concentrations of Cr and Ni (CV 43 to 57%) (Adamo et al., 2008a); the epilithic moss, more exposed to soil and rock dust, had only Cd, Mg and Zn concentrations higher than epiphytic samples.

Different authors argue in favour of the use of apical portions of similar size (Fernández & Carballeira, 2000; Gailey & Lloyd, 1986b; Gill et al., 1975; Little & Martin, 1974) because the exclusive use of such portions eliminates a source of variability and ensures comparable exposure

times. However, no authors have argued in favour of the use of whole shoots. There are two disadvantages associated with the use of entire shoots. Firstly their variable size implies that the capacity for accumulation will also vary due to the heterogeneous nature of the material, and secondly identification of the green part is somewhat subjective.
The availability of green shoots of similar size and with similar concentrations of elements and capacity for bioconcentration, would be another advantage associated with the use of moss cultured in the laboratory.

2.3. Pre-exposure treatment and vital state

Once the material has been selected it can be treated or not treated prior to exposure. As the vital state of the moss (live or dead) will depend on the prior treatment, both aspects will be considered together. The aim of such treatment is to obtain transplants with similar and well defined initial features (similar morphology, well-characterized initial contents of contaminants, comparable physiological status; Giordano et al., 2009), and to maximize the bioconcentration capacity (Gailey and Lloyd, 1986b). In 19% of the papers reviewed, no treatment was carried out. By their nature or aim, some of these treatments (acid washing and/or oven drying) lead to devitalization of the moss. When the moss is subjected to some type of pre-exposure treatment, in 78% of the cases the moss samples are exposed in a live state, and in 32 % the moss samples are devitalized. The sum of these percentages is greater than 100% because both options are sometimes used in the same study.

2.3.1. Washing with water

When pre-exposure treatments are carried out, independently of the vital state of the moss when exposed, the treatment generally includes washing with water (72%). The objective of washing are: (i) to clean the moss of edaphic particles and/or plant remains, in order to have a more homogeneous initial concentration of elements, as reported by Tretiach et al. (2007) and Adamo et al. (2008a), and (ii) to partially activate the tissues, by removing some elements bound to cation exchange sites on the cell wall and membrane (Fernández et al., 2010).
The outcome of the washing step is determined by diverse parameters: (i) number of washes; (ii) duration of washing; (iii) use (or not) of shaking; (iv) type of water, and (v) relation between the weight of the moss and the volume of water. However, of all of the papers reviewed, information about all of these parameters was only reported in one (Tretiach et al., 2007), whereas in 42% of the papers, none of the above parameters was specified. The number of washes, the most specific aspect (53%), ranged between 1 and 7, with the most common being 3 (47%) and 7 (22%). The duration was only detailed in 10% of the studies, and was extremely variable (ranging between 1 minute and overnight), and also varied when more than one washing step was included. Shaking during washing was only applied in 12% of the studies, and details of the intensity were not provided. The type of water was not usually specified (25%), but when it was specified was usually distilled (80%) or bidistilled (13%). In one study, methanol was used in addition to distilled and bidistilled water to wash the samples (Strachan & Glooschenko, 1988). Finally, in the few studies in which the weight of the moss portions in relation to the volume of water was specified, it was 100 g dw per 10 L of water (Adamo et al., 2007, 2008a,b; Giordano et al., 2009, 2010; Tretiach et

al., 2007, 2011). Taking into account all of the above, there is not sufficient evidence to enable selection of the optimal values of the parameters considered.

In any case, even when moss was water washed before exposure and therefore supposed to perform as a living organism, it has been shown to die during exposure, as a consequence of dry and stressing environmental conditions (Adamo et al., 2003; Giordano et al., 2005); accumulation of S, N and C gaseous pollutants was greatly influenced by biomonitor vitality (Vingiani et al., 2004).

2.3.2. Devitalizing treatments

The use of live or devitalized moss depends on the objective of the particular study, which may be to biomonitor atmospheric contamination or to study the effects of such contamination on moss. For the first objective, devitalization enables the efficiency of contaminant capture to remain constant, as capture is mainly due to passive uptake processes that are independent of the vitality of the moss (Aničić et al., 2008 a,b; Basile et al., 2009; Giordano et al., 2009). These processes also include the cation exchange capacity and the capacity for particle retention. The latter is of great interest in urban and industrial areas where particulate forms predominate in emissions (Castello, 2007), and the technique is frequently used in such areas. On the other hand, the use of devitalized samples has some advantages over the use of live moss: (i) from a practical point of view, moss bags can be pre-prepared and therefore readily available at all times; (ii) the results will be less variable (Adamo et al., 2007; Castello, 1996; Gailey and Lloyd, 1986d; Giordano et al., 2009), and (iii) moss metabolism will not affect the results (Giordano et al., 2009). In the latter respect Fernández et al. (2010) have recently demonstrated that growth of *P. purum* during the exposure period affects metal uptake and generates differences in the concentrations of elements finally determined after exposure, independently of the contaminant uptake during the exposure period.

Although some attention had already been given to devitalizing treatments in the past (Castello, 1996; Gailey & Lloyd, 1986b; Tavares & Vasconcelos; 1995), this has been increased in recent years (Adamo et al., 2007, 2008a, 2011; Giordano et al., 2009; Tretiach et al., 2007).

2.3.2.1. Acid washing Acid washing (also called activation) is the devitalizing treatment most commonly used (25% of all studies and 78% of the studies in which the moss samples are devitalized). This treatment consists of immersion of the selected moss material in an acid medium, with the aim of leaching metal ions from the cell walls (Adamo et al., 2007; Castello, 1996; Gailey and Lloyd, 1986d), and disrupting biological membranes (Brown and Brown, 1991; Brown and Wells, 1988). This regenerates (i.e. converts into hydrogen (H^+) form) the cation exchange sites present on the cell wall, with the aim of increasing the bioconcentration capacity of the exposed moss. Adamo et al. (2007) showed that for *H. cupressiforme,* acid washing can reduce the Pb present by up to 96%, with respect to oven dried samples, and by 94% with respect to samples washed with water. The results of the acid treatment are determined by various parameters, as also noted for washing with water: (i) number of washes; (ii) duration of washing; (iii) inclusion (or not) of shaking; (iv) acid used and concentration, and (v) ratio between the weight of the moss and the volume of the acid solution. In 26% of the studies in which the samples are washed in acid solutions, none of the

above parameters were specified. The most common method is a single washing step, and in only 11% of the studies, the samples were washed 3 times. As regards the duration of the washing step, this was only specified in 19% of the studies, and was extremely variable, ranging between 1 and 96 hours in total (including all washing steps). No studies specified whether the samples were shaken during the treatment. The most commonly used acid is HNO_3, at concentrations varying between 0.025 and 1 M, with 0.5 M as most common concentration (54% of the studies), followed by HCl, at concentrations varying between 0.01 and 0.5 M, with the latter most commonly used in the studies where this was specified (80%). With the only exception of Adamo et al. (2007), where 1 mL of 1 M HNO_3 solution was used for 2 mg of moss material, no other studies specified the ratio between the weight of the moss and the volume of the acid solution.

2.3.2.2. Oven drying. The use of oven drying as a method of devitalizing the moss is much less common (7 % of all studies and 23% of studies in which moss is devitalized) and consists of maintaining the material in an oven at a high temperature (>100° C) for 24 hours. Although the aim of this treatment is to devitalize the moss, the moss may also be activated by this treatment as some of the metals may be volatized due to the high temperature. In 86% of cases, the recommendation of Giordano et al. (2009), to dry the moss at 120° C for 24h, was followed. In some studies in which temperatures of between 40 and 50° C were used (Dmuchowski & Bytnerowicz, 2009; Rivera et al., 2011), it was not clear if the aim of the drying process was to devitalize the moss. The advantages of this treatment over acid washing have been described by Giordano et al. (2009): (i) it is eco-friendly, and (ii) it leaves the particular morphology of the moss and leaflet arrangement virtually unaltered, without altering their capacity for capture, whereas the acid notably deteriorates the moss, as it causes rupture of the tissues. However, it has the disadvantage that for some mosses such as *Sphagnum*, the shoots become brittle and the small leaf fragments tend to fall off the stem. In our experience, drying mosses inside the mesh bags by increasing the temperature gradually, minimizes the loss of material. Another disadvantage is that drying does not release metals bound to cation exchange sites as acid washing does. This may be resolved by the use of chelating agents such as EDTA, penicillamine and/or dimercaprol (Pérez-Llamazares et al., 2010) prior to washing with water (see section 1.3.1.). EDTA has been used already by Lodenius & Tulisao (1984) as a chelating agent.
When different pre-exposure treatments (water washed, acid washed and oven dried material) of *Hypnum cupressiforme* were compared in terms of heavy metals accumulation efficiency, no evident trends were observed among them, i.e. it was not possible to identify "the best" treatment . This behaviour was associated to a basically unchanged surface texture after oven and acid pre-treatments, given that surface interception of air-borne particulate is likely to play the major role in accumulation for moss transplants in urban environment (Adamo et al., 2007; Tretiach et al., 2007).

2.3.3. Drying moss samples

In many of the studies reviewed (41%), the final step in preparing the material prior to producing the moss bags consists of drying at low temperature (ranging from room temperature to 40° C), which does not cause death of the moss. The aim of this drying procedure is to remove excess moisture that may remain on the moss

after washing with water or acid (see sections 1.3.1 and 1.3.2.1.). In almost all of the papers reviewed, no information was given about either the temperature or the duration of the drying step.

3. Preparation of the transplants

Preparation of the moss transplants usually involves placing the moss in some type of support (e.g. a mesh net bag), although in some cases moss mats are transplanted (7% of studies). In the latter case, preparation of the transplants, handling of which should be minimal, consists of extracting a portion of the moss from a certain area (usually unpolluted zones) and transplanting it in an other area (usually contaminated zones). The transplanted moss can be placed within some type of frame, e.g. made of wood (Naszradi et al., 2004) or polyethene (Boquete et al., 2011b), or can be placed among the native vegetation without using any type of frame (e.g. Huttunen et al., 1981). Such transplants are often used to evaluate the degree of adaptation of native moss to the contamination in a particular area (see e.g. Kosior et al., 2008, 2010; Tabors et al., 2004). The aim of such studies is therefore different from that of moss bag studies and the methodology is more similar to that used with native moss monitoring.

When preparation of the transplants involves placing the moss in a mesh net bag, the results obtained will be affected by the characteristics of the bag. Such characteristics include: the composition and size of the mesh net, the form and size of the bag and the use of autoirrigation systems. The different alternatives for each of these characteristics, as reported in the literature, are reviewed in the following sections.

3.1. Mesh net material

The composition of the mesh net material is usually specified (91%), and is usually some type of plastic such as nylon (71%), polypropylene (5%), polyethene (3%) or non specified plastic (17%). The materials are usually produced commercially for diverse uses such as hair nets or mosquito nets. Selection of materials such as plastic or glass fibre are more suitable than other less frequently used materials such as cotton (3%) or metal (1%), which may minimally interfere with the uptake process.

The mesh net is sometimes washed with dilute acid before preparing the transplants, to remove trace contaminants (Fernández et al., 2000), especially when mosquito nets are used, as this is sometimes impregnated with chemical insect repellent products. Finally, the bags are usually closed with nylon fibre.

3.2. Mesh size

Of the papers reviewed, 45% did not specify the mesh size. In the remaining papers, the distribution of the mesh size (in size classes, mm2) is extremely variable: <1 (4%), 1-2 (36%), 2-4 (14%), 4-5 (28%), 4-10 (6%), 10-100 (4%), 100-150 (8%), and 150-250 (2%). Selection of the size is a compromise between maximization of the interception of aerial deposition and minimization of the risk of loss of material. Inadequate selection of the mesh size may lead to loss of large amounts of moss, quantified by Strachan & Glooschenko (1998) for *Sphagnum* as between 50 and 75% of the initial amount, as a result of wind action on the mesh bags (1.5x2.0 mm). The thickness of the mesh may also affect the capture of contaminants by the moss, as the mesh may intercept and retain particulate material (Adamo et al., 2008b; Archibold, 1985; Cameron & Nickless, 1977; Zechmeister et al., 2006a).

3.3. Moss bag shape

Although a wide variety of shapes of moss bags are used, these can be summarised in 3 categories: spherical moss bags (36% of studies), flat, square or rectangular moss bags (23%), and cylindrical moss bags (3%). The shape of the bag was not specified in 39% of the papers reviewed. The advantage of three-dimensional bags (i.e. spherical and cylindrical) is that they allow uniform collection efficiency from all directions, whilst permitting collection by gravitational sedimentation (Little and Martin, 1974). In the bidimensional bags (i.e. flat moss bags), exposure of the moss to the atmosphere is more uniform and capture of elements from the atmosphere is improved by the use of some type of system to prevent displacement of the moss further inside the bag (e.g. by sewing the bag so that it contains compartments). This prevents the moss from being flattened on the bottom of the bag under certain conditions (e.g. rain, wind, etc.). The only study in which these types of bags are compared was that carried out by Gailey & Lloyd (1986d), who concluded that the moss in spherical bags captured higher concentrations of metals.

The shape of the bags, in autoirrigated transplants (see section 3.1.) is determined by the type of autoirrigation system used. This system is sometimes included within the bag (e.g. Couto et al., 2004a).

3.4. Quantity of moss employed and size of bags

In order to plan an experimental design using moss bags, several points should be taken into account: i) to verify the availability in natural environment of sufficient moss material with reference to the experimental design (i.e. number of point measures planned); ii) to assure enough material due to analytical constraints, also in view of iii) the loss of material during the exposure. In this last way Temple et al. (1981) reported an average weight loss of 15% in moss *(Sphagnum* sp.) due to laboratory handling and deterioration in the field.

As for the moss quantity to use for preparing each bag, Gailey and Lloyd (1986b) suggested the optimal amount of 100–200 mg but, in order to assure enough material for chemical analysis, the possibility to repeat it and the problems arising from handling, loss of material during exposure, a grater amount should be preferred. To face these problems, the quantity of 400 to 500 mg for each bag has been frequently adopted (i.e. Adamo et al., 2003; Tretiach et al., 2007; Giordano et al., 2009). According to Adamo et al. (2007), the efficiency in metal accumulation of different biomaterials could be better compared expressing the element content of biomonitor on a surface basis instead than on a weight basis; therefore, comparable amounts of different moss species in terms of specific surface area, expressed in $m^2\ kg^{-1}$, could be of help in defining the quantity to employ.

Once established the amount of moss material to be used, the size and shape of the bag has to be decided. The size of the bag determines the ratio between the weight of the moss and the bag surface area. This ratio will affect the efficiency with which the transplants capture contaminants from the atmosphere. However, this information is given in only one of the 112 studies reviewed (Lodenius & Tulisao, 1984), and in most studies (58%) the ratio could not be calculated. In those studies in which the ratio could be calculated (mg cm^{-2}), it was fairly similar : <40 (76%), 40-70 (9%), 70-110 (9%) and >110 (5%).

The optimal size must enhance moss interception and uptake capability as well as replicability for most contaminants. Different authors (Gailey & Lloyd, 1986a; Ze-

chmeister et al., 2006b) recommend that moss should be loosely packed in as thin a layer as possible so that moss shoot overlapping and compression is minimised and is evenly exposed to the contaminants. In fact, when the shoots overlap, the concentrations will decrease gradually. In this respect, Temple et al. (1981) exposed flat bags with moss weight/bag surface area ratios of 10, 20, 30 40 and 50 mg cm^{-2}, and found that the bags with a weight/surface area ratio of 30 mg cm^{-2} were the most suitable for the maximum uptake.

3.5 Bag storage

After preparing the transplants, these are stored waiting to be exposed in the study area. There is not much information about moss bag storage in the bibliography, but it is recommend keeping the moss bags in individual sealed plastic bags (i.e. zip-lock system) to prevent loss of material and avoid moss contamination.

4. Exposure

Once prepared, the transplants will be exposed using different systems of e.g. irrigation, shading and cover, type of support, location, height and duration of the exposure and number of bags exposed. The combination of all these options will affect the final concentration of contaminants in the moss. As several different combinations are reported in the literature, options will be considered separately.

4.1. Irrigation systems

Moss samples are mostly exposed live (not devitalized) (in 65% of the studies reviewed). An irrigation system was used to keep the moss alive during the period of exposure in only 23 % of such studies. Although some authors consider the use of non irrigated transplants to be valid (e.g. Cameron and Nickless, 1977; Hynninen, 1986; Lodenius and Tulisalo, 1984), many others have shown the opposite. The main problem is that these bags tend to dry out and thus their efficiency in retaining metals will vary depending on environmental conditions (Giordano et al., 2009), such as air humidity, precipitation, solar radiation, wind intensity, etc. (Tyler 1990). In some studies, higher concentrations of metals have been detected in wet seasons than in dry seasons (Adamo et al., 2003; Giordano et al., 2009; Tavares & Vasconcelos, 1996). Clough (1975) found higher deposition velocities in dry moss bags than in wet moss bags since the more open structure of dry moss bags provides a larger effective surface area for deposition, but that for particles of > 5 µm diameter, deposition velocities are higher in wet moss bags due to reduced bounce-off of particles from the wet surface. Some authors have reported death of the moss during the exposure period (Basile et al., 2009; Goodman & Roberts, 1971; Huttunen et al., 1981; Tavares & Vasconcelos, 1996; Tretiach et al., 2007;). This would lead to a change in the capacity of the moss to capture contaminants at some time during the exposure period, thus preventing comparison of the results obtained under different environmental conditions (i.e. in time and space). In this view, Vingiani et al. (2004) demonstrated that, differently from trace elements that are accumulated by moss transplants mainly through passive processes of retention, the accumulation of S, N and C by mosses is mainly based on an active process of intake of pollutants in gaseous forms.

The alternative to using live, non-irrigated, transplants is to use irrigated transplants. As already indicated, metal uptake is affected by the moss metabolism, and exposure of the irrigated moss is only recommended when the aim is to study biological variables (e.g. enzyme activity, photosynthesis,

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etc.). Two types of irrigation systems can be applied. The first, autoirrigation, was first introduced by Al-Radady et al. (1993); the system consists of placing the moss on a capillary mat connected to a recipient, generally made of plastic, full of water. In some studies the moss is placed directly on a capillary mat and both are placed in a mesh (Amblard-Gross et al., 2002; Couto et al., 2004a), while in other studies the mesh bag containing the moss is placed on the capillary mat (e.g. Al-Radady et al., 1993; Aničić et al., 2009b). Higher concentrations have been detected in autoirrigated *Sphagnum girgensohnii* than in dry ones (Aničić et al., 2008), possibly because the deposition velocity of particles is much higher on wet moss bags than on dry ones (Clough, 1975). The second type of irrigation system, possibly less practically applicable, is to spray the moss bags with distilled water weekly (Basile et al., 2008) or twice a week (Mariet et al., 2011).

4.2. Shading systems

In six of the papers using autoirrigated transplants, these are placed in a steel frame and covered with a shading net, to reduce environmental and hydric stressors, such as direct solar radiation (reduced by up to 70%) and wind (Aboal et al., 2008; Couto et al., 2004a,b; Fernández & Carballeira, 2000; Fernández et al., 2010; Skert et al., 2009). The aim of removing environmental stressors is to enable clearer identification of relationships between the concentrations of certain contaminants in moss tissues and their effect on different physiological variables. On the other hand, use of this type of shading net varies the inputs of contaminants relative to the exterior, and also generates a higher degree of heterogeneity within the steel frame (due to e.g. dripping points).

4.3. Shelter systems

In a number of cases, when the objective is to quantify only the dry deposition (11% of the studies reviewed), shelters or covered sites (e.g. below balconies; Rivera et al., 2011) are used to prevent exposure of the moss bags to wet deposition and/or loss by washing out of some of the contaminants retained (Couto et al., 2004a; Zechmeister et al., 2006a,b). Several different types of systems are used to cover the bags, including PVC sheets (Fabure et al., 2010), plastic containers such as plant pots (Lodenius 1998; Sun et al., 2009) and inverted funnels (Xiao et al., 1998).
The use of covers to prevent exposure of the moss from wet deposition does not necessarily imply that the dry deposition can be quantified accurately. The presence of a cover also prevents the largest particles reaching the moss, and may alter the dynamics of particle deposition (Little, 1977). Likewise, the covers will also alter the form and intensity with which the wind affects the moss samples (Tavares & Vasconcelos 1996), which will also influence bioconcentration of the contaminants (e.g. loss of previously retained particles).
Only one study has used high side sheltering (Amblard-Gross, 2002), which allows vertical atmospheric deposition onto the moss from the open circular area around the moss, and prevents lateral capture influence from the wind.

4.4. Positioning of moss bags

The way in which the moss bags are positioned will only affect flat bags, as uptake occurs in all directions in spherical bags (Goodman & Roberts, 1971). The position of flat bags must be considered in relation to the ground (i.e. horizontal or vertical) and the focal point of contamination. In most studies based on flat bags, these are positioned vertically, which

enables capture of contaminants present in the air (gases and small particles with low sedimentation rates) from the horizontal wind flow into which they faced. Horizontal positioning of bags, which is less common (in 33 studies), increases the capture of contaminants by processes of gravitational sedimentation and wet deposition (Goodman et al., 1975). The latter requires installation of some type of complex support (see section 3.5.), which is a practical disadvantage compared with the vertical arrangement for which the bags are usually positioned using simpler systems.

As regards the orientation of the bags relative to the source of contamination, this is only important in vertically positioned bags, and is not mentioned in most studies. The orientation of the bags relative to the source of contamination has been taken into account when using transplants to monitor isolated focal points of contamination. This enables the flat surface of the bag to be directed towards the source being monitored (Temple et al., 1981). Gailey and Lloyd (1986b) showed that the replicability of the results obtained with vertical *Sphagnum sp.* bags, with acid washed treatment, that were not oriented exactly perpendicular to the main source of airborne metals was lower than that of results obtained with perpendicularly oriented bags. As with horizontally positioned bags, some type of support must be used to orientate vertically positioned bags, which is a practical complication. In light of the above, it is clear that for vertically positioned bags, the orientation of the bags in relation to the focal point of contamination must be taken into account (i.e. perpendicular to the focal point or another specific orientation).

4.5. Location and type of support used

The moss bags should be placed as far as possible from obstacles (e.g. buildings, vegetation, etc.) that may interfere in the exposure of the moss to atmospheric contaminants. Once the site is selected, the moss bags are suspended (e.g. by a nylon fibre) at a certain height (see section 3.6) on different structures (e.g. trees, lamp posts, etc.), by use of different types of supports. The supports (e.g. plastic tubes, glass fibre poles, etc.) should be made from inert materials that are not affected by the contaminants under study, and do not release contaminants during exposure. They should function to maintain the moss bags separated from each other and as far as possible from structures, in order to prevent effects such as screening or dripping. Although the moss bags were suspended from vegetation in 15 of the studies, this option should not be used as vegetation is not inert, it may shield the moss from atmospheric contaminants and also cause a concentration effect by dripping from leaves and twigs onto the bags from above (Little & Martin, 1974).

In some cases, more complex supports (see section 3.4.) from which to suspend moss bags have been used (Tretiach et al., 2007; Makholm & Mladenoff, 2005; Temple et al., 1981). For example, Temple et al., (1981), constructed a special U-shaped support in which the rectangular moss bags are inserted into a sleeve, and a notch in the support prevents this holder, and therefore the moss bag, from turning about its vertical axis, thus allowing the flat surface of the bag to be directed towards the source being monitored. Two complex types of latticeworks, one round of galvanized steel and the other rectangular and made by plastic sticks and nylon ropes, were realised to expose bags on the roof of automated

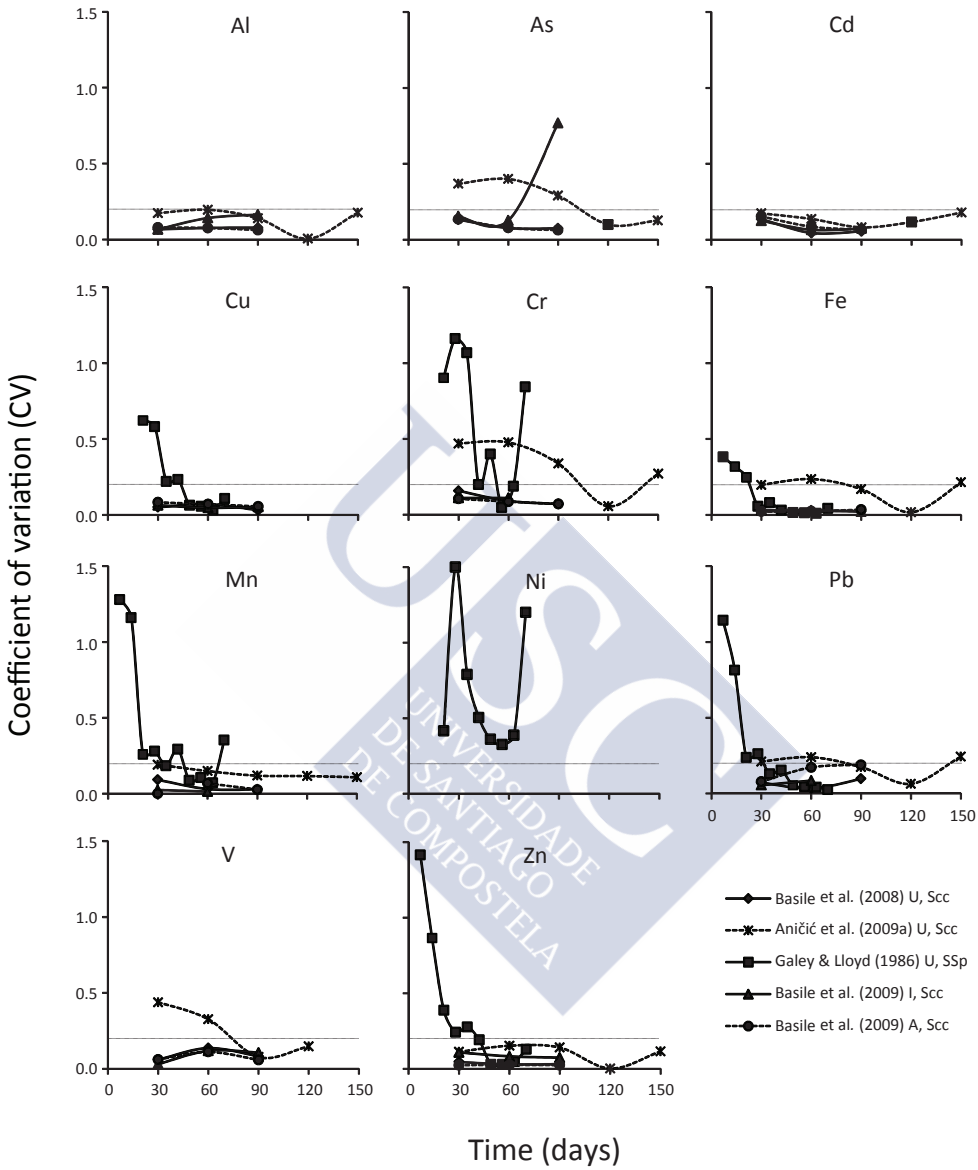


Fig. 4. Changes in the coefficients of variation (CV) of the concentrations of contaminants in different studies with moss bags over time for each metal. The following are indicated in the legend, beside each study, the number of replicates used in each study, the species employed and the area where the study was carried out. In each graph, the level corresponding to the 20% error is represented by a line of points. (I=industrial; A=agricultural; U=urban; Scc= *Scorpiurum circinatum*; Sg= *Sphagnum girgensohnii*; SSp=*Sphagnum sp*).

air pollution monitoring stations located in Trieste and Naples urban areas. Various bags of different biomaterials including moss, were arranged on each latticework in order to expose them at the four cardinal points and avoiding contacts (Tretiach et al., 2007).

4.6. Height of exposure

There is a huge variability as regards the height of exposure of the moss bags, ranging from ground level (see e.g. Huttunen et al., 1981; Samecka-Cymerman & Kempers, 2007) to almost 30 m (Culicov et al., 2005; Rivera et al., 2011). The information is usually provided (82% of studies) and varies as follows: <4 m (82%); 4-7.5 m (9%); 7.5-11.5 m (4%), and >11.5 m (5%). The differences in height affect the results obtained as air-flow and turbulence vary enormously with height above the ground and have substantial effects on the amounts of contaminants collected (Adamo et al., 2011). The latter authors found that at low heights (4 m), the contamination detected in a narrow street canyon in the urban area of Naples city is related to emissions from traffic and suspended dust from the roads, whereas at higher heights (12 and 20 m) the movement of air masses favours accumulation of elements originating from long-distance transport and cations of marine origin.

Diverse factors must be taken into consideration when selecting the height of exposure: the objectives of the study (Little and Martin, 1974), e.g. 2 m to assess the quantities of contaminants inhaled by people from the air; or practical aspects, such as availability of bag holding facilities and necessity to suspend the bags at a sufficient height to prevent loss through vandalism.

4.7. Duration of exposure

There is also a great deal of variability as regards the time of exposure of the moss bags, despite the fact that this has been one of most frequently studied aspects (see e.g. Basile et al., 2009; Gailey & Lloyd, 1986d; Ratcliffe, 1975; Tremper et al., 2004; Vasconcelos & Tavares, 1998). The duration of exposure ranges between 1 week (see e.g. Tavares & Vasconcelos, 1996) and 20 months (Evans & Hutchinson, 1996). This information is provided in almost all studies (98%) and varies as follows: <1 month (13%); 1-<2 months (41%); 2-<3 months (23%); 3-<6 months(16%), and >6 months (7%).

Ratcliffe et al. (1975) proposed a series of criteria in order to determine the most appropriate length of exposure for the accumulation of most contaminants: (i) detectable accumulated concentrations; (ii) reliable values (i.e. high replicability), and (iii) an exposure time within the limits of practical considerations. Another fundamentally important criterion which would be advisable to add to this list is that concentration in the moss increases almost linearly over the exposure period.

In order to evaluate the above criteria, the moss bags were exposed for different periods of time after a common starting point in several studies (28%). The replicability generally tends to increase with the time of exposure of the moss transplants. For studies in which a minimum of three exposure periods were tested, the changes in the coefficients of variation (CV) of the concentrations of contaminants are shown in Fig. 4. In general terms, irrespective of other factors (e.g. species, pre-exposure treatment, shape of bag, etc.), no trends in the CV were observed after 30-45 days of exposure, and the variations tended to be lower for higher concentrations (usually < 20%). The largest fluctuations in the CV were observed for contaminants present at low concentrations (i.e. Ni and Cr; Gailey & Lloyd, 1986d; Fig. 4). However, the

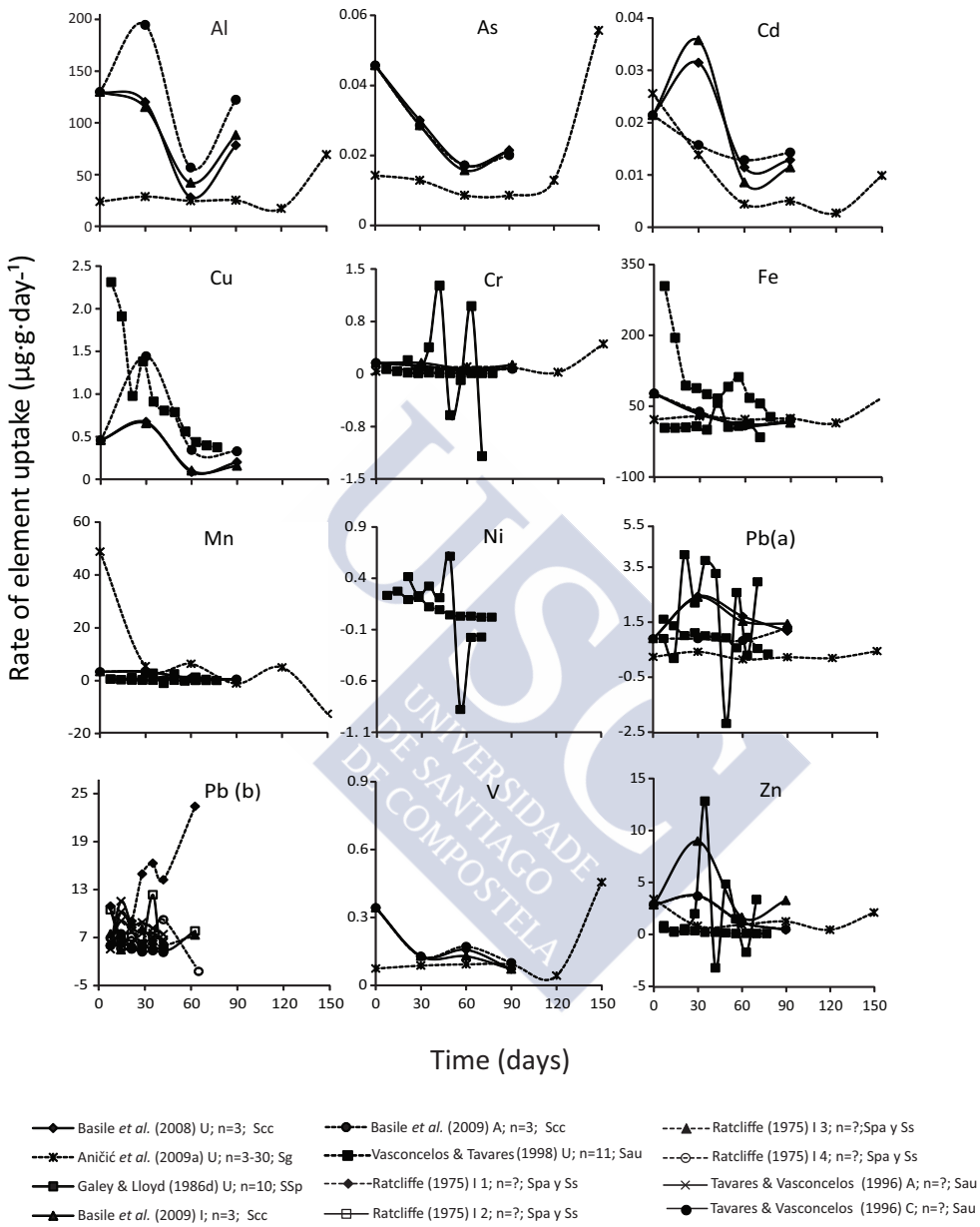


Fig. 5. Changes in the rate of uptake (µg g day^{-1}) of different contaminants in different studies with moss bags. The following are indicated beside each study: the area where the study was carried out, the number of replicates employed in each study and the species used. As numerous studies determined the concentrations of Pb, the data are included two graphs. (U=urban; I=industrial; A=agricultural; C=control; Sau= *Sphagnum auriculatum*; Scc= *Scorpiurum circinatum*; Sg= *Sphagnum girgensohnii*; Spa= *Sphagnum papillosum*; Ss= *Sphagnum subsecundum*; SSp= *Sphagnum spp.*).

Table 1. Calculation of the number of replicate moss bags required for sampling errors of 10, 15 and 20% respectively, for different studies considering different contaminants and an exposure period of 30 days. An asterisk indicates n> 50, and --- indicates that the modal value could not be calculated. (L= live moss; F= flat moss bag; Ps= *Pleurozium schreberi;* Pp= *Pseudoscleropodium purum;* Ep= *Eurhynchium praelongum*; Up= Unpolluted; Sg= *Sphagnum girgensohnii*; U= Urban; D= devitalized moss; Sc= *Sphagnum cristatum;* I= Industrial; Am= acid washed moss; Sa= *Sphagnum angustifolium;* S= spherical moss bag; Scc= *Scorpiurum circinatum;* R=Rural; SSp= *Sphagnum sp;* Hs= *Hylocomium splendens;* T= Traffic; B= moss bag; Bc= *Bryum capillare)*.

References	Details	Al	As	Ba	Be	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Se	Sr	V	Zn
Amblard-Gross et al.,2002	L,F,Ps,Pp,Ep,Up			1,1,1		1,1,1								1,1,1		2,1,1	1,1,1	
Anicic et al.,2009b	L,F,Sg,U	12,5,3	*,23,13	16,7,4	21,9,5	12,5,3	12,5,3	26,11,6		15,7,4	7,3,2	14,6,4		17,8,4	1,1,1	10,5,3	*,25,14	5,2,1
Archibold and Crisp, 1983	D,F,Sc,I,Am	7,3,2					43,19,11	4,2,1	39,17,10	9,4,2		3,1,1	*,27,15	18,8,5			5,2,1	*,*,31
Archibold, 1985	D,F,Sa,I,Am								2,1,1	2,1,1		7,3,2		2,1,1				1,1,1
Ares et al., 2011	L,F,Pp,U-I,								3,1,1									
Basile et al.,2008	L,S,Scc,U	2,1,1	7,3,2			7,3,2		10,4,2	1,1,1	1,1,1		3,1,1		2,1,1			1,1,1	1,1,1
Basile et al.,2009	L,S,Scc,I	2,1,1	9,4,2			6,3,2		7,3,2	2,1,1	1,1,1		1,1,1		4,2,1			1,1,1	5,2,1
	L,S,Scc,R	2,1,1	7,3,2			9,4,2		4,2,1	3,1,1	1,1,1		2,1,1		3,1,1			1,1,1	1,1,1
Fernández and Carballeira, 2000	L,F,Pp,I						*,23,13	*,25,14	16,7,4					22,10,5	*,*,*			7,3,2
Fernández et al., 2000	L,F,Pp,I							*,*,28			*,*,33		5,2,1					*,35,20
Gailey and Lloyd,1986b	L,F-S,SSp,U-I,Am							*,*,*	*,*,32	1,1,1		30,13,8	*,*,*	27,12,7				23,10,6
Little and Martin, 1974	L,S,SSp,I					7,3,2								10,4,2				5,2,1
Santamaria and Martin, 1997	D,S,SSp,Up,Am					5,2,1			3,2,1	2,1,1		28,12,7		29,13,7				28,12,7
Tan et al., 2000	D,S,U,Am										*,*,*							
Zechmeister et al., 2006b	L,F,Hs,T	*,24,13	*,33,19	26,11,6		20,9,5	20,9,5	*,23,13	*,26,15	*,27,15	*,*,35		38,17,9	1,1,1			*,26,15	44,20,11
Zhang et al., 2009	L,B,Bc,Up	9,4,2						*,*,35	15,7,4	6,3,1		11,5,3	22,10,5	23,10,6				13,6,3
	L,B,Bc,I	8,4,2						*,43,24	4,2,1	9,4,2		4,2,1	13,6,3	12,6,3				
Modal value		2,1,2	7,3,2	---	---	7,3,2	---	4,2,1	3,1,1	1,1,1	---	3,1,1	---	2,1,1	---	---	1,1,1	5,2,1

Table 2. Calculation of the number of replicates required for errors of 10, 15 and 20% respectively, for different studies considering various contaminants and an exposure period of 30-60 days. When An asterisk indicates n> 50, and --- indicates that thee modal value could not be calculated.(L= live moss; F= flat moss bag; Ps= *Pleurozium schreberi;* Pp= *Pseudoscleropodium purum;* Ep= *Eurhynchium praelongum*; Up= Unpolluted; Sg= *Sphagnum girgensohnii*; U= Urban; I= Industrial; S= spherical moss bag; Scc= *Scorpiurum circinatum;* R=Rural; D= devitalized moss; Hc= *Hypnum cupressiforme*; Am= acid washed moss; C= Control; Sp= *Sphagnum palustre;* SSp= *Sphagnum sp;* Hs= *Hylocomium splendens;* Sca= *Sphagnum capillifolium*; St= *Sphagnum teres* T= Traffic*)*.

References	Details	Al	As	Ba	Be	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Se	Sr	Zn
Amblard-Gross et al., 2002	L,F,Ps,Pp,Ep,Up			1,1,1		1,1,1								1,1,1		1,1,1	
Anicic et al.,2009b	L,F,Sg,U	15,7,4	*,27,15	18,8,4	15,7,4	7,3,2	24,11,6	32,14,8		21,10,5	7,3,2	9,4,2		22,10,6	5,2,1	13,6,3	9,4,2
Ares et al., 2011	L,F,Pp,U-I		*,32,18			*,49,28					19,9,5		*,*,32	*,*,30			
Basile et al.,2008	L,S,Scc,U	2,1,1	2,1,1			1,1,1		3,1,1	2,1,1	1,1,1		1,1,1		1,1,1			1,1,1
Basile et al.,2009	L,S,Scc,I	8,3,2	6,3,2			2,1,1		5,2,1	1,1,1	1,1,1		1,1,1		1,1,1			3,1,1
	L,S,Scc,R	2,1,1	2,1,1			3,1,1		3,1,1	2,1,1	1,1,1		1,1,1		2,1,1			1,1,1
Castello, 1996	L-D,S,Hc,I	1,1,1				26,11,6		30,13,7	20,9,5	1,1,1		*,*,*	15,7,4	23,10,6			
	L-D,S,Hc,I,Am	12,5,3				38,17,10		42,19,11	11,5,3	15,7,4		8,4,2	37,16,9	18,8,5			
	L-D,S,Hc,Up	8,3,2				13,6,3		*,*,30	31,14,8	12,5,3		14,6,4	42,19,11	*,26,14			
	L-D,S,Hc,C,Am	25,11,6				*,46,26		30,13,8	*,32,18	8,4,2		*,*,33	*,*,32	*,*,31			
Castello, 2007	L-D,S,Hc,I	*,*,*	*,34,19			*,*,*		*,*,*	*,*,*	*,*,*	*,*,43	*,*,*		*,*,*			*,*,31
	L-D,S,Pp,I	23,10,6	*,*,*			*,*,*		*,*,*	*,*,*	*,*,*	*,*,41	*,*,*		*,*,*			*,*,36
Fernández and Carballeira, 2000	L,F,Pp,I						*,34,19	*,*,37	29,13,7					*,*,*	*,*,*		30,13,7
Fernández et al., 2000	L,F,Pp,I																
Gailey and Lloyd, 1986d	D,S,Sp,U-I,Am							*,*,*	*,*,*	*,*,34		*,*,*	*,*,*	*,*,*			*,*,*
Gailey and Lloyd, 1986d	D,S,SSp,U-I,Am									2,1,1			22,10,6	3,1,1			
Gailey and Lloyd,1986b	L,S-F,SSp,U-I							1,1,1	1,1,1	1,1,1		4,2,1	41,18,10	1,1,1			1,1,1
Little and Martin, 1974	L,S,SSp,I																
Lodenius, 1998	D,S,SSp,I,Am										*,*,*						
Rivera et al., 2011	L,F,Hs,U	*,*,*	*,*,*			*,43,24		*,*,39	*,*,*					*,*,*			31,14,8
Yurukova and Ganeva, 1997	L,F,Sca,Up	1,1,1	1,1,1			1,1,1	1,1,1		1,1,1	1,1,1		1,1,1	1,1,1	1,1,1			1,1,1
	L,F,St,Up	1,1,1	1,1,1			1,1,1	1,1,1		1,1,1	1,1,1		1,1,1	1,1,1	1,1,1			1,1,1
	L,F,Sca,I	1,1,1	19,9,5			7,3,2	1,1,1		5,2,1	1,1,1		1,1,1	2,1,1	2,1,1	6,3,2		3,2,1
	L,F,St,I	1,1,1	26,12,7			8,4,2	1,1,1		6,3,1	1,1,1		2,1,1	11,4,2	2,1,1	8,3,2		3,1,1
Zechmeister et al., 2006b	L,F,Hs,T																
Modal value		1,1,1	1,1,1	---	---	1,1,1	1,1,1	3,1,1	1,1,1	1,1,1	---	1,1,1	1,1,1	1,1,1	3,3,2	---	1,1,1

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most marked decrease in the CVs corresponded to the shortest exposure periods (i.e. 30-45 days for Cu, Fe, Mn, Pb and Zn; Gailey & Lloyd, 1986d; Fig. 4) reaching below 20% for most elements.

For studies in which a minimum of three exposure periods were considered, and without taking into account other factors such as the species etc., we have plotted the rate of uptake of contaminants against the time of exposure (Fig. 5). The uptake of contaminants by the moss was not found to be linearly related to the exposure period. Moreover, the rate rarely depended on time (see as exceptions; Cu and Fe in Vasconcelos & Tavares, 1998; Cd and Mn in Aničić et al., 2009a), and the greatest changes were observed during the first exposure period. The concentration of contaminants in the moss tissues often increase with the exposure time, although there is sometimes a negative relationship (Cr, Ni, Pb and Zn; Gailey & Lloyd, 1986d). The lack of a relation between the rate of uptake and the time of exposure may depend on diverse factors (Aničić et al., 2008). Firstly, there may be differences in the atmospheric levels of the contaminants during the different exposure periods. Secondly the weather conditions (e.g. wind, rain, etc.) may affect the availability (e.g. via solubilization) and/or loss of contaminants (e.g. by leaching caused by excess rain, detachment through wind, cation displacement, etc.) and may also modify the uptake capacity of non irrigated moss samples (see sections 1.3.2. and 3.1.). For example, during 6-weeks exposure periods in the same urban environment but in the spring season of two consecutive years the element accumulation by *Hypnum cupressiforme* increased during wetter weather and higher PM_{10} conditions (Giordano et al., 2009). The same authors observed that moss bags exposed for a 12-week period accumulated higher element concentrations than the sum of those accumulated in two consecutive 6-week periods, indicating that in the study area (with low pollution levels) neither 6- or 12-week exposure causes saturation of the biomatrix capacity to retain elements.

On the basis of the above, an exposure period of between 30 and 45 days is proposed, as this will ensure accumulation of elements, adequate replicability of the results, and is practically acceptable (and in fact is the most commonly used period in the literature consulted), and finally because when there is a relationship between the uptake and exposure time, the slope of the corresponding regression line is usually steepest within this period.

4.8. Number of bags

The number of moss bags exposed by way of replicates in active biomonitoring studies is very variable and ranges between 1 and 30. In most studies, replicates are not used (39% of the studies), followed by use of 2 or 3 replicates (28%), 4 (13%), 5 (7%), 6-<15 (9%) and >15 (4%).

One way of selecting the most suitable number of moss bags is to consider the level of error allowed in estimating the mean concentrations of contaminants in the moss tissues. If it is assumed that the concentrations are normally distributed (unfortunately this information is not available in studies in which n>30; Aničić et al., 2009a) and that the mean and standard deviation are known, the number of replicate bags that will provide a certain percentage of error can be calculated ($N=(t^2\ \sigma^2)/(D2\ \mu2)$, were σ^2 and μ are the variance and mean of the data; D is the level of error assumed and t in the value of the statistics in a Student´s t test at the 5% significance level). The number of bags required for errors of 10, 15 and 20%, calculated from data available in the literature, for exposure periods of 30 and

Table 3. Variables in the methodology of the moss bag technique used to monitor the air quality, and studies carried out with the aim of optimizing these variables, the options most commonly used, and finally the recommendations for a harmonised protocol, is proposed based on the information available

Variable	Optimization	Option most commonly used	Recommendations
1. Preparation of the moss			
1.1. Selection of species	*Sphagnum* sp[e]	*Sphagnum* sp. (51%)	*Sphagnum* sp. preferiblemente from a cloned culture.
1.2. Selection of material	Needs further research	Apical portion (19%)	Green apical portions (5 cm)
1.3. Pre-exposure treatments and vital state	Devitalized	Live moss (63%)	Devitalized
1.3.1. Washing with cellular extractants	Devitalized		
1.3.1.1. Number of washes		---	1 wash with EDTA (10 mM) + 1 with Dimercaprol (30 mM)
1.3.1.2. Duration of washing		---	20 minutes
1.3.1.3. Shaking		---	Wash with shaking
1.3.1.4. Weight of moss per volume of extractant		---	1 L water per 12 g dw
1.3.2. Washing with water	Needs further research	Washed with water (72%)	Water washed
1.3.2.1. Number of washes		3 times (47%)	3 times
1.3.2.2. Duration of washing		---	20 minutes
1.3.2.3. Shaking		---	Wash with shaking
1.3.2.4. Type of water		Distilled water (80%)	Distilled water
1.3.2.5. Weight of moss per volume of water		10 L of water per 100 g dw	10 L water per 100 g dw
1.3.3. Devitalizing treatment	Oven drying, 24 h 120ºC [a,b]	Acid washed (25%)	Oven drying, 24 h 120ºC
2. Preparation of transplants			
2.1. Mesh material	Inert material	Nylon mesh (71%)	Nylon mesh
2.2. Mesh size	Large without loss of material	1-2 mm (35%)	2 mm
2.3. Shape of bag	Spherical [c]	Spherical moss bags (35%)	Spherical (ensuring single layer of moss)
2.4. Size of bag	12 mg cm^{-2} [e]	<40 mg cm^{-2} (32%),	12 mg cm^{-2}
3. Exposure			
3.1. Shading system	General heterogeneity	no	No conclusion
3.2. Cover	Underestimation of deposition	no	No conclusion
3.3. location and type of support used		----	Free of obstacles, suspended from inert support by nylon thread
3.4. Height of exposure	4 m[e]	< 4 m (82%)	4 m
3.5. Duration of exposure	8 weeks[e]	4-<8 weeks(41%)	8 weeks
3.6. Number of bags for site		1 (39%)	3 bags
3.7. Initial concentrations and controls		---	3 controls and 3 initial times
4. Post exposure treatment	No treatment	No treatment (72%)	No treatment

[a] Fernández et al. (2010); [b] Giordano et al. (2009); [c] Gailey and Lloyd (1986d); e present study

30-60 days respectively (chosen on the basis of the information in section 3.7) are shown in Tables 1 and 2. In some cases the number of bags is extremely high, much higher than 50 (i.e. n> 4000 for an error of 10%), due to the large difference in the concentrations in the bags exposed together. For some elements, such as Cr, Hg and Pb, several replicate bags (i.e. n> 10) are required for both periods, unlike for other elements such as Al, Fe, Mn, etc. In general terms, the concentrations in the moss bags exposed for periods of 30-60 days were less variable than those exposed

for 30 days, as indicated by the modal values shown in both tables. The maximum modal numbers of bags for all elements, corresponding to sampling errors of 10, 15 and 20%, were 7, 3 and 2 for a 30 day exposure period, and 3, 3 and 2 for an exposure period of 30 - 60 days, respectively.

4.9. Initial and control concentrations

In most studies (64%), part of the material destined for use in the moss bags is conserved to determine the initial concentrations of each of the contaminants under study. These concentrations are then used to calculate enrichment factors (EF= final concentration/initial concentration) and the net enrichment (NE= final concentration minus initial concentration) for each of the transplants and contaminants studied. It is also considered important to apply certain measures to prevent contamination of the samples outside the exposure period or exposure site. Gailey & Lloyd, (1986b) found that the replicability of the results decreased greatly when strict guidelines for sample handling were not followed, and therefore that handling of samples during preparation, collecting, transport to the laboratory and preparation for analysis, can cause different degrees of contamination or of loss of adhering particles. Some studies included control bags, which were subjected to all handling and transportation steps, but which were not exposed in the study area (Ares et al., 2011; Couto el al., 2004b). In Giordano et al. (2009) for the preparation of each bag, samples of c. 700 mg of different biological materials were taken; 200 mg were used as subsamples for initial concentration analysis, the remaining 500 mg were put into the bag for exposure. For each biomaterial 12 bags were prepared and analysed to assess the variability of initial concentrations, which, for most of the elements, was lowest in water washed moss and highest in acid-washed moss.

5. Post exposure treatments

Once the moss has been exposed, the samples may be washed in the laboratory prior to being prepared for analysis (in 18% of the studies reviewed), with washing times that range from a few seconds (Samecka-Cymerman & Kempers, 2007) to 10 minutes (see e.g. Fernández et al., 2000). The aim of this is to remove weakly bound elements or those bound on particles deposited on the surface of the moss and to evaluate the effect of rainfall events during exposure. No research has been carried out to optimize the washing of devitalized moss after the exposure period, or to determine how effective the procedure is or whether it alters the extracellular equilibrium.

As regards the use of live moss, some authors (Wells & Brown, 1990) have indicated that washing times of more than 30s may alter the extracellular equilibrium, thus causing loss of at least part of the contaminants bound to the cell wall and the external face of the membrane, so that long washing times should not be applied. On the other hand, Aboal et al. (2011) have recently demonstrated, by scanning electron microscopy, that in samples of native *P. purum*, washing for 30s is ineffective for removing the particles. For moss in bags, the post-exposure water washing reduced consistently the concentrations of most accumulated elements and induced further leakages of the metabolic elements (K, Mg, Mn, Ni) already reduced during exposure (Giordano et al., 2009). The same authors showed that the post-exposure water washing reductions were always more consistent in treated materials (acid-washed and oven-dried) compared with untreated ones reinforcing the convince-

ment that in areas affected by low rainfall rates, most elements are accumulated in biomonitors as particulate matter whose heterogeneous nature reflects natural and anthropogenic sources.

6. Conclusions and final remarks

It is clear that there is a lack of standardization of the moss bag technique and this hampers the routine use of this technique as a regular tool for environmental monitoring by official institutions. The variables associated with this technique for monitoring air quality are summarised in Table 3. These variables are far from being fully investigated; nevertheless in Table 3, taking into account the studies so far carried out to optimize these variables and the options most commonly applied we propose a harmonised protocol.

When the objective of the use of moss transplants is a physiological study, irrigated transplants should be used, so that the proposed protocol would not be applicable. As regards the species that should be used, the information available to date indicates that the most commonly used species are those of the genus *Sphagnum*. However, isolation and cloning of a particular species of terrestrial moss would provide more homogeneous material. The cloning aspect is perhaps the most urgent as regards the definition of a standardised methodology. Nonetheless, it is clear that in whichever of these cases, only a fixed size of green apical portion of the shoots should be used to prepare the transplants. Pre-exposure treatments will determine the vital state of the moss at the time of exposure; if live moss is used, we recommend that it is washed with water following Tretiach et al. (2007). If devitalized moss is used, the material should be treated with cellular extractants (EDTA and dimercaprol) to free cation exchange sites, then washed with water and finally oven dried in gradual stages to devitalize the moss minimising tissue fragmentation. As regards preparation of the moss bags, nylon net with a 2 mm mesh size is the proposed material. It is inert, does not interfere with the uptake process, and the mesh size should ensure minimal loss of the moss during the exposure period. Spherical bags are the most commonly used, and have been shown to enable capture of a higher concentration of contaminants than flat bags. However, we recommend further studies to identify a system for maximising the surface area of moss exposed to the air. An example could be the use of two concentric spherical bags made from a rigid mesh enabling exposure of a single layer of moss that will not become compacted within the bag. Moreover, unlike bidimensional (flat) bags, spherical bags are not affected by the direction in which they are placed (i.e. the orientation relative to the focal point of contamination).

The bags should be located away from any obstacle that may interfere in the accumulation of contaminants in the moss, so that vegetation should not be used to support the bags. Although it is not yet clear at what height the bags should be placed, it has been demonstrated that the exposure period should be between 30 and 45 days. Exposure of 3 replicate bags at each sampling site for this period guarantees a sampling error of less than 10%.

We wish that after this review a large number of studies will adopt the proposed harmonised protocol in order to test it in different environmental cases. Optimization of the different variables could stem from parallel experiments aimed to assess the degree to which their modification will affect the final result. It should

be considered that some of the variables described may interact with each other, so that an iterative optimization process may be required. Taking into account the objectives proposed in the Introduction, the optimization process should mostly lead to obtain a greater replicability of the results. At the state it appears difficult to achieve another of the proposed objectives, i.e. a linear relationship between the concentrations of the elements captured by the moss and the concentration in the atmosphere. This is what is most frequently requested to biomonitors to represent a valid alternative to conventional monitoring technique. However moss bags do not act as integrators of contaminants and often show negative rates of contaminant uptake. Their chemical composition is the result not only of additive uptake but also of loss of accumulated elements by leaching. Therefore they cannot exhibit linear relationships with atmospheric depositions. They should be rather employed in addition to conventional monitoring, offering the possibility of a lower cost, more flexible and dense monitoring design able to put in evidence spatial and temporal trends, vertical and horizontal gradients of a wider variety of inorganic and organic pollutants.

Acknowledgements

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General objectives

1. To carry out a quantitative evaluation of the effect of moss vitality, and thus of growth, on the uptake of heavy metals during the exposure period (Chapter I). For this purpose, self-watering, shaded moss transplants were used, as this enables the physiological properties of the moss to be held constant.

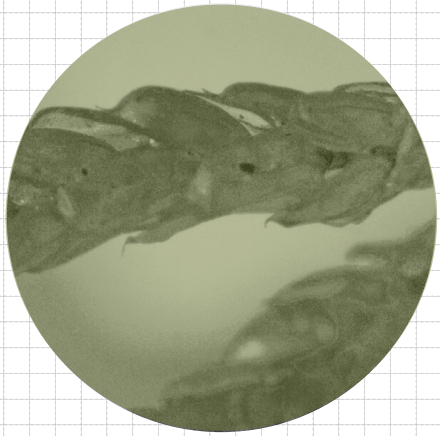
2. To optimize key methodological aspects in the application of the moss bag technique (Chapter II) having into accounto the provisional protocol proposed in the "State of the art" section by the selection of the optimal option for: i) selection of species, ii) the relationship between moss weight and bag surface area, iii) duration of exposure and iv) height of exposure on the basis of the replicability and final concentration data (Cd, Cu, Hg, Pb and Zn). For this purpose, the present study was carried out at different sites affected by different degrees of contamination, and different exposure periods were used.

3. To investigate whether the levels of contaminants measured in moss are correlated with the concentrations in bulk deposition (Chapter III). To achieve this objective, the study was carried out with standardized moss bags located in different sites affected by different degrees of contamination, for different exposure periods.

4. To validate the technique by studying the spatial patterns of dispersion of contaminants (heavy metals and PAHs) in an industrial area, an urban area and an area affected by heavy road traffic (Chapter IV).

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Chapter I



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Effect of growth on active biomonitoring with terrestrial mosses

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Abstract

The effects of growth of autoirrigated, shaded transplants of *Pseudoscleropodium purum* on the quantification of tissue concentrations of Cd, Cu, Hg, V and Zn, were investigated in 4 exposure periods, each of 56 days, at 7 sampling sites (contaminated and uncontaminated). Concentrations of the elements in the basal portions of the moss shoots were compared with the concentrations in the portions of the shoots that grew during the exposure period. Mercury and V were present at lower concentrations in the new portions of the shoots than in the basal portions, whereas the opposite was true for Cd, Cu and Zn. The magnitude of error introduced by growth was not negligible, and in some cases was higher than 40%, relative to the results obtained by analysis of the whole shoot. Devitalization of moss prior to its use as transplant material is recommended to avoid growth of the plant during the exposure period.

Keywords: Pseudoscleropodium purum, transplants, heavy metals, contamination, pollu tion.

1. Introduction

In the period since moss transplants were first used to monitor air quality (Goodman and Roberts, 1971), the technique has been successfully used in industrial (see e.g. Aboal et al., 2008; Couto et al., 2004; Fernández et al., 2000; Lodenius and Tulisalo, 1984; Pilegaard, 1979) and urban environments (see e.g. Adamo et al., 2007; Aničić et al., 2009a; Naszradi et al., 2007; Vasconcelos and Tavares, 1998). Transplanted "moss bags" are often used rather than native moss because of: i) lack of native moss in an area, especially where levels of contaminants are relatively high or where climatic and environmental conditions are unsuitable (Giordano et al., 2009); ii) elimination of possible phenotypic and genotypic adaptations of native moss to toxicity in contaminated environments (Bargagli, 1998); and iii) the use of autoirrigated, shaded transplants homogenizes the bioconcentration conditions and removes possible environmental stressors (i.e. moisture, radiation, etc.), thereby enabling evaluation of the specific effects of contaminants on moss physiology (e.g. Aboal et al., 2008; Amblard-Gross et al., 2002). However, we are unfortunately still far from having standardized protocols, and therefore methodological procedures must be optimized, with the emphasis on data quality (Adamo et al., 2007, 2008;

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Giordano et al., 2009; Tetriach et al., 2007). The lack of such protocols hampers comparison of the results obtained in different studies, and sometimes limits the conclusions that can be reached.

Various studies have shown that active biomonitoring should involve transplants in similar, well defined initial conditions, and in recent years particular attention has been given to key steps of the technique, such as prior treatment of non irrigated samples (by washing with water or acid, or drying in an oven; Adamo et al., 2007, 2008; Giordano et al., 2009; Tetriach et al., 2007). These authors concluded that devitalization of the samples by oven drying is the best method, for various reasons: i) relative to other methods of devitalization (e.g. acid-washing) it is eco-friendly; ii) the variability in the pre-exposure concentration of elements is low and comparable to that of the water-washing preparation procedure, and the moss thus selected for bag preparation reduces the variability in biomonitoring results; iii) it shows the least variability in post-exposure concentration of elements relative to the other two methods, with no differences in the values obtained with the water-washed mosses, thus improving the results obtained; iv) it leaves the particular morphology of the moss and leaflet arrangement virtually unaltered, thus enhancing capture of metals as particles from the atmosphere; and finally and perhaps most importantly; and v) it prevents interference with element uptake due to moss metabolism, which appears to be exclusively passive and based on surface interception.

However, until now the latter aspect has not been well studied and it is not known how moss metabolism contributes to uptake or, more specifically how growth of the moss during the exposure period affects uptake. Transplanted live moss may grow if the environmental conditions are suitable. The portion that grows will obviously have been exposed to the atmosphere for less time and therefore the concentration of elements in this tissue may be lower than in the older portions of the shoot. Growth of moss will vary depending on differences between seasons and geographical location, which may generate differences in the concentrations of elements measured, independently of the load of contaminant taken up during the exposure period.

The aim of the present study was to test the hypothesis: "there is not interference/ contribution of growth of transplanted mosses as regards uptake of Cd, Cu, Hg, V and Zn during the exposure period". Traditional moss bags are not suitable for this type of study, as metabolism and growth of the moss samples will not be maintained if environmental conditions are not suitable. Autoirrigated, shaded transplants were therefore used, as the moss physiology is maintained during the exposure period (Aboal et al., 2008).

2. Material and methods

2.1. Sampling

The study was carried out in Galicia (NW Spain), at seven sampling sites (SS): (i) three (SS1-SS3) were located in the surroundings of a coal-fired power plant (1400 MW) at distances of between 10 and 20 km, and were orientated in different directions; (ii) two (SS4-SS5) were located in the same way in the surroundings of another coal-fired power plant (550 MW); (iii) one (SS6) was located close to an aluminium smelter (less than 1km); and finally (iv) one (SS7) was located close to a station included in the European Monitoring and Evaluation Programme (EMEP) Network. Stations SS1 to SS5 have atmospheric heavy metal burdens of Cd, Hg, V and Zn (Aboal et al, 2006; Real

et al, 2008); in SS6 there is atmospheric deposition of Cu, Hg, V and Zn (Fernández et al., 2007); and SS7 corresponds to a background station.

2.2. Preparation of transplants

Samples of the terrestrial moss *Pseudoscleropodium purum* (Hewd.) M. Fleisch were collected from a rural area isolated from urban and industrial centres. In the laboratory, 3 cm long apical segments were separated from the shoots. In order to determine the error associated with measurement of the apical segments, 100 such segments were chosen at random on different occasions, and measured with a digital caliper; the mean length obtained was 3 ± 0.1 cm (mean ± 95% confidence interval for the mean). The samples were sieved on a plastic mesh (0.7 cm) and then washed three times - each time for 1 min - with shaking (the first time with distilled water and then with double distilled water) to remove plant remains, epiphytes, adhered edaphic particles, etc. The washing procedure partially activates the moss as it removes elements bound to the cation exchange sites in the cell wall and membrane. Excess moisture was removed from the samples by blotting them with filter paper, and 5 g (fresh weight) of the material was weighed out for preparing the transplants. Some aliquots of the material were set aside as samples to achieve initial concentrations, and were vacuum packed during the exposure period to prevent them being contaminated.

Moss apices were placed on a capillary mat placed on top of a plastic container; the short ends of the mat were inserted through slits positioned close to the edge of the lid of the container. The container was filled with distilled water, so that the moss was maintained at high relative humidity, to prevent hydric stress. The moss, capillary mat and the lid of the container were covered with plastic mesh (mesh size 0.5 cm), to ensure that the moss remained in contact with the water and also to prevent it being blown away by the wind. In the field, three such transplants were placed inside a stainless steel frame completely covered with a plastic shading net, to reduce the effect of direct solar radiation and to provide protection against the wind. This type of shade shelter would decrease the concentration of metals at the moss surface. The frames were placed at a height of 2 m in each SS, away from any obstacles and exposed to winds from all directions.

The transplants were exposed in situ, for 56 days. After the material was removed it was replaced with new transplants. The study began on 12 December 2008 and ended on 28 July 2009, so that by the end of the experiment a total of four sets of transplants had been exposed at each SS.

Once in the laboratory, the moss transplants were washed with double distilled water, with shaking, for 30 s - to avoid altering the equilibrium of the extracellulary bound cations (Wells and Brown, 1990). The lower 3 cm of the transplant, corresponding to the material present from the start of the exposure period (first portion – Por.1) was separated from the part of the apical segment that grew the during the exposure period (second portion – Por.2). The samples were dried at room temperature, then homogenized in an ultracentrifugal mill (Restch ZM 200, heavy metal free) and dried at 80º C before analysis; an aliquot was dried at 40º C for determination of Hg.

2.3. Chemical analysis

The concentrations of Cd, Cu, V and Zn were determined in solid suspensions by graphite furnace atomic absorption spectroscopy (Perkin Elmer AAnalyst 600). Two measurements were made for each

sample. The concentrations of Hg were determined in an elemental analyser (Milestone DMA 80).

To control the analytical quality, analytical replicates were processed, 1 every 10 samples (1 each sample for Hg), and the standard deviation was calculated according to Cêburnis and Steinnes (2000). Certified reference material was analysed in parallel - M2 and M3 corresponding to the moss *Pleurozium schreberi* (Steinnes et al., 1997) and an internal reference material of *P. purum* - once every 10 samples. Contamination during processing was controlled for by the use of analytical blanks, 1 every 10 samples analysed.

2.4. Statistical procedures

As most of the variables were not normally distributed, as determined by application of the Shapiro-Wilk test, Wilcoxon's test was used to test for differences between the concentrations in the initial portion (present from the start of the exposure period) and those in the portion that grew during the exposure period. For the same reason, Spearman's correlations were used to analyse the relationship between the percentage growth of each of the transplants and the difference in concentrations of the elements in each portion.

3. Results

3.1. Analytical quality

The concentrations were all higher than the corresponding limits of quantification. Recoveries of the different reference materials used ranged between 84 and 110% for Cd, 94-120% for Cu, 76-96% for Hg, 93-132% for V, and 88-118% for Zn. The error associated with the determination of Cd, Cu and Hg was below 5%, and for V and Zn, below 10%.

3.2. Differences in the concentrations of heavy metals in relation to growth of the moss

The elements selected could be determined in all transplants in both studied segments (n=21, for each segment and in each period). However, as a result of poor growth of Por.2 of the moss samples in SS7, the sample size was smaller in the 3rd period for Hg (n=19) and V (n=20), and in the 4th period for V (n=20) and Zn (n=20).

No growth was observed in the first two periods of exposure, so that it was not possible to determine the concentrations in the apical segments that grew during the exposure period. During winter, at these latitudes, the growth of *P. purum* is the minimum along the year. Unfortunately, in the studied winter the growth was really negligible. The percentage growth (weight Por.1/weight Por.2) in the 3rd and 4th periods is shown in Table 1, and was higher in the 4th period.

The mean concentrations determined in both apical segments (Por.1 and Por.2) for each site and period, as well as the mean confidence interval of 95% are shown in Table 1 and Fig. 1. The results for each element were similar in both periods, with minimal variations. Likewise, the concentrations of Cd, Cu and Zn in Por.2 were highly variable, unlike those in Por.1, whereas for Hg and V, the variability was similar in both segments, or even higher in Por.1 in the 4th period. In all cases, the variability was higher at higher concentrations, independently of the portion considered (Por.1 or Por.2).

The results of the Wilcoxon's test revealed significant differences between Por.1 and Por.2 in all cases ($Z=-4.015$, $p<0.0001$ for Cd and Cu in the 3rd and 4th periods, Hg in the 4th period and Zn in the 3rd period; $Z=-3.920$, $p<0.0001$ for V in the 3rd and 4th and Zn in the 4th, and $Z=-3.139$, $p<0.002$ for Hg in the 3rd period).

However, although the differences in concentrations between the two periods were significant for all elements, the sign differed. For Cd, Cu and Zn, the concentrations in Por.2 were higher than those in Por.1. In all cases, the concentration in Por.2 was higher than the initial concentration (horizontal line in the graph in Fig. 1), but in the cases of Cd and Zn the concentrations in Por.1 were greater or equal, with some exceptions, than the initial concentration, whereas the concentration of Cu in Por. 1 was always lower than or equal to the initial concentration. Inversely, for Hg and V the concentrations were lower in Por.2 than in Por.1. For both metals, the concentration in Por.1 was higher than the initial concentration, but the concentration of Hg in Por.2 was higher than the initial concentration, unlike for V.

The results of the Spearman's correlations between the percentage growth of each of the transplants (weight Por.1/weight Por.2) and the difference in concentration (concentration Por.2 – concentration Por.1) was significant for Cu in both periods (r=-0.792*** and r=-0.426*) and for Hg (r=-0.567** and r=-0.440*) and Cd in a single period (r=-0.436*). Thus for Cd and Cu, as the differences in concentrations were positive and the correlation negative, growth of the moss during the transplant period was inversely related to the differences in concentration, this is, the higher the growth the lower differences. However, in the case of Hg, as the differences were negative and the correlation negative, the higher growth corresponds to the greater differences.

The percentage error due to growth of the apices during the exposure period is shown in Table 1. For this, the concentration that would be obtained if both portions were analysed together was estimated (i.e. [weight Por.1/(weight Por.1 + weight Por.2)]*concentration in Por.1 + [weight Por.2/(weight Por.1 + weight Por.2)]*concentration in Por.2); this estimate was expressed as a percentage of the concentration in Por.1 (i.e. (estimated concentration /concentration in Por.1)-1). This provided an estimate of the error introduced by growth due to the differences in concentration between the two portions.

4. Discussion

Before starting the experiment it was expected that, if the moss grew, the concentration of elements in the new tissue (Por.2) would be lower than in the original segment because the new tissue would be exposed to the atmosphere for less time than the older tissue. At the end of the experiment it was found that this was generally true for Hg and V, although there were differences in uptake of these elements. In accordance with the starting hypothesis, the longer the exposure period, the greater the uptake, so that the increase in concentration of the elements in the original segment (Por.1) should always be higher than the final concentration in the new segment (Por.2), as was observed for V (Fig. 1). This result is consistent with the conclusions reached by Adamo et al. (2007) and Giordano et al. (2009), i.e. that element accumulation in transplanted mosses (not irrigated) is mainly due to passive processes. However, the results obtained for Hg are not consistent with the latter, as in the 3rd period the concentrations in Por.1 and Por.2 tended to equalize, and in the 4th period the final concentration in Por.2 tended to be higher than the increase relative to the initial concentration determined in Por.1. Such differences in the concentrations of Hg in different periods may be related to the greater growth observed in the 4th period, as for this element, the growth produced an increase in the magnitude of

Table 1. Mean (± 95% confidence interval) percentage growth (% G) and mean concentrations of Cd and Hg (ng g-1), and of Cu, V and Zn (µg g-1) in the 3 moss transplants for each sampling site and period (3rd and 4th), where Por.1 corresponds to the basal 3 cm of the shoot, exposed from the start of the exposure period, and Por.2 corresponds to the apical segment that grew during the same period. The percentage error, introduced by growth of the moss as a result of the differences in concentrations in the two portions, is also shown. (---: not available).

			Cd			Cu			Hg			V			Zn		
Period	**SS**	**% G**	**Por.1**	**Por.2**	**% error**	**Por.1**	**Por.2**	**% error**	**Por.1**	**Por.2**	**% error**	**Por.1**	**Por.2**	**% error**	**Por.1**	**Por.2**	**% error**
3rd	1	27 ± 3	110 ± 28.6	156 ± 32.3	9 ± 2	10.2 ± 1.20	24.5 ± 1.55	30 ± 8	83.0 ± 8.40	72.7 ± 11.5	-3 ± 5	3.26 ± 0.747	0.530 ± 0.194	-18 ± 3	47.5 ± 11.0	79.0 ± 17.8	16 ± 15
	2	32 ± 3	45.5 ± 4.24	103 ± 5.90	31 ± 2	10.4 ± 1.20	19.4 ± 1.41	22 ± 5	58.0 ± 5.00	46.5 ± 2.50	-5 ± 2	1.98 ± 0.147	0.252 ± 0.103	-21 ± 1	35.4 ± 5.96	85.0 ± 7.00	35 ± 9
	3	26 ± 7	49.0 ± 4.55	129 ± 71.7	31 ± 18	11.1 ± 0.616	21.4 ± 2.28	19 ± 6	59.0 ± 0.356	45.0 ± 7.77	-5 ± 3	2.66 ± 0.174	0.798 ± 0.126	-14 ± 3	117 ± 44.0	152 ± 61.6	6 ± 4
	4	19 ± 1	59.2 ± 23.0	103 ± 28.6	13 ± 3	10.5 ± 0.480	30.6 ± 8.10	31 ± 12	51.0 ± 5.00	48.5 ± 4.38	-1 ± 1	2.56 ± 0.269	0.515 ± 0.145	-13 ± 0	35.7 ± 24.8	97.3 ± 30.6	33 ± 15
	5	16 ± 9	44.0 ± 4.45	92.9 ± 2.72	15 ± 8	12.2 ± 2.28	41.8 ± 15.3	30 ± 6	56.0 ± 4.08	56.5 ± 2.76	0 ± 1	2.48 ± 0.246	0.656 ± 0.0656	-10 ± 5	54.5 ± 5.66	103 ± 35.7	12 ± 9
	6	13 ± 2	78.0 ± 12.6	126 ± 17.0	7 ± 0	11.8 ± 1.88	28.9 ± 5.05	17 ± 1	64.6 ± 3.09	61.6 ± 1.68	-1 ± 1	9.19 ± 0.626	3.40 ± 0.389	-7 ± 1	44.4 ± 11.7	110 ± 18.6	19 ± 14
	7	12 ±15	48.0 ± 2.44	103 ± 5.75	10 ± 11	11.2 ± 1.66	28.9 ± 13.5	11 ± 8	57.6 ± 9.90	41.0 ± ---	-4 ± ---	2.10 ± 0.145	0.444 ± 0.0434	-8 ± 8	39.8 ± 15.1	85.9 ± 1.90	15 ± 21
4th	1	24 ± 7	68.7 ± 16.2	123 ± 57.6	15 ± 10	8.18 ± 0.780	15.0 ± 0.840	16 ± 4	89.9 ± 14.5	69.0 ± 8.00	-4 ± 2	3.33 ± 1.07	0.838 ± 0.117	-14 ± 5	74.7 ± 27.3	121 ± 43.8	12 ± 7
	2	36 ± 3	43.4 ± 3.90	102 ± 79.0	37 ± 52	9.20 ± 0.545	12.2 ± 0.660	9 ± 3	88.9 ± 9.80	44.5 ± 4.28	-13 ± 1	2.32 ± 0.808	0.283 ± 0.189	-23 ± 3	53.0 ± 13.9	91.0 ± 24.2	19 ± 5
	3	32 ± 10	56.6 ± 13.3	68.7 ± 6.00	7 ± 9	10.9 ± 4.20	16.7 ± 3.46	13 ± 11	65.0 ± 3.40	40.4 ± 2.48	-9 ± 2	2.76 ± 0.970	0.697 ± 0.066	-17 ± 4	115 ± 50.0	162 ± 50.5	13 ± 12
	4	35 ±18	53.0 ± 13.3	78.0 ± 22.4	10 ± 10	8.18 ± 1.06	12.5 ± 2.02	13 ± 2	82.8 ± 5.25	42.5 ± 5.96	-13 ± 7	3.88 ± 1.90	0.384 ± 0.224	-23 ± 9	50.0 ± 7.50	102 ± 8.79	26 ± 7
	5	34 ± 12	47.5 ± 16.8	63.6 ± 13.7	9 ± 4	10.4 ± 2.20	16.4 ± 8.10	15 ± 15	91.9 ± 11.6	44.4 ± 5.96	-13 ± 2	4.24 ± 1.35	0.570 ± 0.280	-22 ± 5	52.5 ± 22.4	88.0 ± 19.4	19 ± 11
	6	21 ± 2	72.7 ± 2.70	103 ± 3.60	7 ± 0	10.3 ± 0.490	20.8 ± 8.30	18 ± 13	98.0 ± 14.2	75.7 ± 9.20	-4 ± 1	7.78 ± 2.16	6.06 ± 0.687	-4 ± 4	63.6 ± 14.9	109 ± 15.8	13 ± 8
	7	12 ± 7	45.5 ± 0.398	72.7 ± 0.768	6 ± 3	9.40 ± 0.626	14.8 ± 2.54	6 ± 4	91.0 ± 12.1	59.6 ± 5.20	-4 ± 3	2.14 ± 0.194	0.299 ± 0.172	-7 ± 1	68.7 ± 6.30	80.2 ± 2.56	1 ± 0

the differences in the concentrations between the two segments (Por.2 - Por.1). A second hypothesis to explain the different behaviour showed by Hg can be related to the fact that this element could be deposited in gaseous form while the other studied elements occurring in atmospheric deposition as ions or particles.

The pattern of uptake of Cd, Cu and Zn was different from that of the latter two metals, and was not consistent with the initial hypothesis. For these elements in all periods and seasons, the final concentrations (Por.2) were always higher than the concentrations in Por.1. These results are consistent with those reported by Aničić et al. (2009b), who (in a comparison of metal uptake in irrigated and non irrigated transplants) observed higher concentrations of Hg and V in non-irrigated moss than in the irrigated moss (unlike what they observed for the other metals studied); the authors did not offer any explanation for their findings.

In light of the results for Cd, Cu and Zn, and to a lesser extent Hg, an alternative hypothesis may be suggested. 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; Aničić et al., 2009a) may take place in addition to passive processes. Therefore the active uptake process, which appears to operate exclusively during the formation of new tissues, also appears to be much more rapid than the passive process (Fig. 1), as although the new segments (Por.2) are exposed for a shorter time, the final concentrations were higher than in the original material (Por.1). Furthermore, in accordance with Couto et al. (2003), the concentration in the transplanted moss reflects a balance that is determined by the inputs and outputs of metals in the tissues during the exposure period. Thus in the case of Cu and Zn, on some occasions the final concentration in the transplanted segment (Por.1) was lower than the initial concentration (Fig. 1), which demonstrates a negative balance in the metal concentrations during the exposure period. Despite this negative balance in the original material (Por.1), the final concentrations in the new tissue (Por.2) were in all cases much higher than the initial concentrations (corresponding to mosses that grew and developed in an unpolluted environment), and in some cases up to three times higher (i.e. Cd and Zn in Fig. 1). This appears to indicate a higher capacity for bioconcentration during the period of formation of new tissues, which may maximize capture of tissue constituents. If there are high concentrations of other elements in the environment, some will also be incorporated into the tissues, as occurs with all of the elements studied, except for V. This process was observed for Cu and Zn, which of the elements under study, reach the highest concentrations in *P. purum* by natural means (Carballeira et al., 2002). The incorporation of metals in new tissues (Por.2) is sufficiently strong to prevent their loss (by leaching, cation competition or anionic complexation), unlike in the tissue that did not grow during the exposure period (Por.1), perhaps indicating inclusion of the metals in the intracellular compartment or as tissue components. These results are consistent with the differences between the apical zone and basal shoots of *P. purum* reported by Leblond et al. (2004), i.e. higher apical concentrations of Zn and lower apical concentrations of Hg. Although other authors (Bargagli et al., 1995; Bargagli, 1998) found similar concentrations of Hg in brown parts (old) and in green parts (young) for the same species, being slightly higher in the brown. A second alternative hypothesis would be that new moss seg-

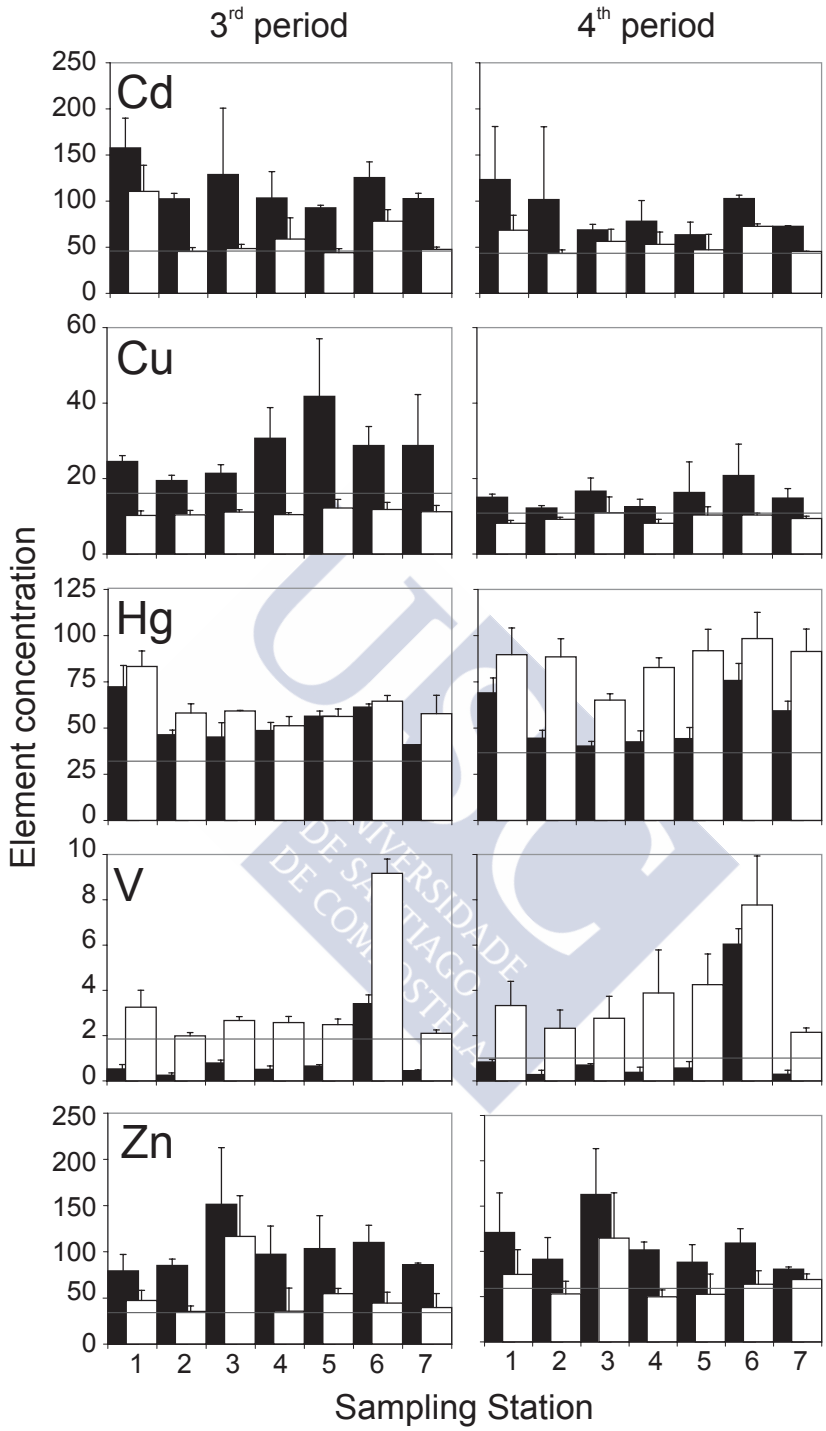


Fig. 1. Mean concentrations (vertical lines represent 95% confidence interval) of Cd and Hg (ng g^{-1}), and of Cu, V and Zn (µg g^{-1}) in the 3 moss transplants for each sampling site and period (3rd and 4th). White bars represent the basal 3 cm of the material exposed from the start of the exposure period (Por. 1),and black bars represent the apical segments that grew during the same period (Por. 2). The horizontal line represents the initial concentrations.

ments are softer (i.e. they have less cellulosic material) hence have less density, determining as a consequence higher metal concentrations. Finally, a third alternative can be related to the movement of essential elements for plant metabolisms towards the active apical segment, according with results reported by Loppi et al., (1997) in lichen *P. caperata*, which found higher concentrations of Zn and Cu in the new parts of the tallus (peripheral zone) than in the old (central zone).

The present results demonstrate that the previously mentioned processes are produced in the same way in all scenarios studied, and are therefore independent of the source of contamination, and therefore of the form of metal considered (particulate, dissolved in precipitation or gaseous). Irrespective of the causes and mechanisms of action, the results obtained have evident implications for biomonitoring studies. Growth of transplanted moss during the exposure period was found to play a fundamental role in the uptake process, which is not exclusively passive or only based on surface interception. Aničić et al. (2009a) concluded that the concentrations of elements are less variable in non irrigated moss bags than in irrigated moss bags during different exposure periods, indicating that irrigated moss is more sensitive to changes in the concentrations of trace elements in the atmosphere. However, in light of the present results, such differences may be attributed to different rates of growth at different times of the year. In determining metal concentrations in moss, the error associated with growth of moss during the exposure period, in relation to the concentration that would be obtained without growth (Table 1) is proportional to the growth, which is usually greater in the 4th period. Such errors result in overestimation of the concentrations of some metals (i.e. Cd, Cu and Zn), and underestimation of the concentrations of others (i.e. Hg and V), with the magnitude of error ranging between 10 and 20% in most cases. In some cases the errors were greater than 30% and even 40%. Thus, given the different growth rates at different times of year, and the different environmental conditions to which the transplants may be exposed (because of different biogeographical or microclimate locations), the error introduced in determining the concentrations of metals is not at all negligible. Although the results were obtained with autoirrigated, shaded transplants, it is expected that if the environmental conditions during the exposure period are favourable, the transplanted moss will grow and therefore the error will already be introduced as all of the material is analysed together.

Various authors (Aničić et al., 2009b; Al-Radady et al., 1993; Astel et al., 2008) consider that use of irrigated moss bags (or their exposure along with devitalized moss; Szczepaniak et al., 2007) is the most appropriate method, as it improves the efficiency and homogeneity of capture of elements, relative to non-irrigated moss bags, which tend to dry out and thus their capacity to retain elements varies depending on the environmental conditions, especially humidity (Al-Radady et al., 1993). Another reason given for using irrigated transplants is that deposited particles are trapped in greater quantities on wet surfaces, so that wet moss is more likely to incorporate the elements in its tissues, and the elements will be less susceptible to rinsing and better reflect the atmospheric conditions (Aničić et al., 2009c; Astel et al., 2008). However, the present results demonstrate a serious problem (not previously identified) related to growth of the transplanted moss, and therefore support the use of devitalized moss in such studies, as proposed by other authors (Adamo

et al., 2007, 2008; Giordano et al., 2009; Tetriach et al., 2007).

Conclusions

The process of uptake of elements in autoirrigated, shaded transplants of *P. purum* is not always proportional to the length of exposure. This may be due to active processes of uptake that occur during growth of new tissue, characterised by greater speed of uptake and a higher retention capacity. It was therefore concluded that the uptake process is not exclusively passive or only based on surface interception. In addition, the magnitude of the error associated with growth was not negligible (in some cases >40%), and differed in direction depending on the element considered (leading to underestimation or overestimation of the concentrations). The present conclusions support those of Giordano et al. (2009) as regards the need to devitalize the moss samples (by oven-drying) prior to their used as transplant material. Finally, when the objectives of the study include analysis of physiological variables and the moss must therefore be kept alive during the exposure period, the effect of growth must be taken into account for correct analysis of the results.

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Chapter II



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Towards the methodological optimization of the moss bag technique in terms of contaminants concentrations and replicability values.

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Abstract

The moss bag technique is a simple and economical environmental monitoring tool used to monitor air quality. However, routine use of the method is not possible because the protocols involved have not yet been standardized. Some of the most variable methodological aspects include (i) selection of moss species, (ii) ratio of moss weight to surface area of the bag, (iii) duration of exposure, and (iv) height of exposure. In the present study, the best option for each of these aspects was selected on the basis of the mean concentrations and data replicability of Cd, Cu, Hg, Pb and Zn measured during at least two exposure periods in environments affected by different degrees of contamination. The optimal choices for the studied aspects were found: (i) *Sphagnum denticulatum*, (ii) 5.68 mg of moss tissue for each cm^2 of bag surface, (iii) 8 weeks of exposure, and (iv) 4 m height of exposure. Duration of exposure and height of exposure accounted for most of the variability in the data. The aim of this methodological study was to provide data to help establish a standardized protocol that will enable use of the moss bag technique by public authorities.

Keywords: Biomonitoring harmonization, environmental tool, heavy metals, air quality, atmospheric deposition.

1. Introduction

The moss bag technique is the most commonly reported method of active biomonitoring with terrestrial mosses. The technique is a simple and cost-effective way of evaluating air quality, among other advantages mentioned by numerous authors (see e.g. Sun et al., 2009; Rivera et al., 2011; Saitanis et al., 2013). However, use of the moss bag technique remains restricted to the scientific field because of the lack of internationally standardized protocols (see e.g. Little and Martin, 1974; Gailey and Lloyd, 1986a,b,c; Giordano et al., 2009), while, in the case of native moss a norm for biomonitoring air quality exists (CEN/TC 264/WG 31). This lack of standardization hampers the comparison of the results and conclusions of different studies, which

in turn precludes use of the technique by official institutions in the implementation of environmental monitoring programs.

Several well-defined steps must be taken into account while applying the moss bag technique for biomonitoring purposes. These steps mainly involve the selection and preparation of moss, preparation of bags, exposure conditions and post-exposure treatments (Ares et al., 2012). Only a very few of the methodological aspects of the technique have been standardized or investigated in detail (e.g. devitalizing treatment during moss preparation; Giordano et al., 2009). The high degree of variability in each of the methodological steps greatly influences the results obtained. The following are some of the most variable steps (for review see Ares et al., 2012): i) selection of species; ii) ratio between the weight of moss and surface area of the bag; iii) duration of exposure and iv) height of exposure.

In previous studies, selection of the different options involved in the application of the moss bag technique has been carried out on the basis of practical considerations or, in some cases, by taking into account the maximum accumulation of contaminants in the moss tissues. In this way: i) it was demonstrated that *Sphagnum spp*. is able to accumulate higher quantities of contaminants than other commonly used biomonitoring species due to their morphological and physiological characteristics (Temple et al, 1981; Bargagli 1998; González and Pokrovsky 2014); ii) an optimal quantity of moss per surface area is considered that where a thin layer of moss is placed within the bag, with all the shoots with the same exposed surface (Gailey and Lloyd, 1986a; Zechmeister et al., 2006), however Temple et al. (1981) consider than 30 mg cm^{-2} provide the optimal accumulation rates; iii) longer duration of exposure than 1 or 2 weeks are recommended as the contaminant uptake increase with the duration of exposure (Gailey and Lloyd, 1986b) iv) and regarding the height of exposure, 4 m are recommended because of some practical considerations, besides Vuković et al. (2013) pointed out that samples exposed at this height showed higher element accumulation than 8 or 16 m. However, the best option must be selected on the basis of other key requirements (Ratcliffe, 1975; Ares et al., 2012): (i) transplants should be easy to prepare and handle; (ii) they should enable replicable results to be obtained; and (iii) they should be efficient at capturing contaminants, indicating occurrence in the atmosphere within a reasonable time. The present study focuses on optimization of the different methodological steps on the basis of contaminant concentrations and replicability of the results.

The within-site replicability of samples has not been studied in depth in relation to any of the above-mentioned methodological aspects, except the duration of exposure (Gailey and Lloyd, 1986b). As these studies were carried out with non-devitalized moss and as growth of moss during the exposure period affects the metal uptake measured in different portions of the shoot (Fernández et al., 2010), it is advisable to test different times of exposure with devitalized moss (Giordano et al., 2009). However, little attention has been paid to the influence of the type of site on the sample replicability. Indeed, different microenvironments or sites affected by different degrees of contamination (i.e. urban, industrial, rural) may represent important sources of variability (Tretiach et al., 2011).

Investigation of data replicability also enables evaluation of the total variability associated with different methodological steps, and it is possible to rank these aspects in terms of variability. Thus, efforts should be

focused on standardizing the most variable aspects of the moss bag technique.

We previously proposed a standardized protocol (Ares et al., 2012) aimed at providing a starting point for further research, to enable improvement and optimization of the technique. The aim of the present study is to select the best options for different aspects of the technique, on the basis of sample replicability and final concentrations of different elements (Cd, Cu, Hg, Pb and Zn). The different aspects considered were the moss species, the ratio between the weight of the moss sample and bag surface area, the duration of the exposure and the height of exposure. The study was carried out in different sites affected by different degrees of contamination and for different periods of exposure.

2. Material and methods

2.1. Preparation of moss transplants

Moss bags were prepared with *Sphagnum denticulatum* Brid., except in experiment 2.2.1. (see below), in which *Pseudoscleropodium purum* (Hedw.) M. Fleisch. was also used. These are two of the most commonly used species in the moss bag technique (Ares et al., 2012). The moss specimens, both epigeic, were collected in mountain areas: *S. denticulatum* in a pristine wetland and *P. purum* in a rural area selected in basis of previous results (Boquete et al., 2013). Both places were situated at more than 300 m from main roads and populated areas. In the laboratory, the green parts (3-6 cm approximately) of the moss were separated from the rest of the shoot, placed in a plastic sieve (0.7 cm mesh size) and washed 3 times in bidistilled water (10 L water per 100 g of dry weight of moss, 10 min per wash with shaking) to remove plant remains, epiphytes and adhering soil particles. The samples were then blotted on filter paper to remove excess moisture, and around 8 g fresh weight (f.w.) (approximately 1g in dry weight) of tissue was placed in each bag, except in experiment 2.2.2. (see below), in which the weight was

Table 1. Summary of the study area characteristics showing the sampling site codes, geographical coordinates (UTM 29T ED50), altitude (h), description of the area and sampling sites where each experiment was carried out. (SP: selection of species; QS: quantity of moss per unit of bag surface area; DE: duration of exposure and HE: height of exposure).

Coordinates					Methodological aspect			
SS Codes	X	Y	h (m)	Area description	SP	QS	DE	HE
SS1	567178	4817834	26	Steel-works factory	x	x	x	
SS2	567147	4818261	5	Steel-works factory	x	x	x	x
SS3	567938	4818376	7	Steel-works factory	x			
SS4	567447	4818989	9	Steel-works factory	x			
SS5	583842	4837331	288	Coal-fire power plant		x	x	
SS6	486249	4754973	21	Fe-Mn smelter		x	x	
SS7	622773	4839371	13	Aluminia-aluminium smelter		x	x	x
SS8	536390	4747182	232	Periurban		x	x	x
SS9	539494	4747484	303	Highway roadside				x
SS10	566344	4826193	30	Rural area	x			
SS11	616468	4684220	974	Rural area		x	x	
SS12	586485	4710232	694	Rural area		x	x	
SS13	549827	4733561	306	Rural area				x

varied.

Flat bags (400 cm^2, therefore 2.5 mg cm^{-2} except in experiment 2.2.2., see below) were made with fiberglass netting (2 mm mesh size), previously washed in HNO_3 to eliminate any trace contaminants. The moss was distributed homogeneously inside the bag, which was then sewn in a zig-zag pattern with nylon thread to make compartments and minimize overlapping and compression of the moss. The bags were then dried in an oven at 120ºC for 24h to devitalize the moss (Giordano et al., 2009). Finally, the bags were vacuum packed and stored at -20ºC until use.

2.2. Experimental set-up

All the experiments were carried out in Galicia (NW Spain). Different sampling sites (SS) affected by different degrees of contamination were selected for each of the experiments (i.e. industrial, peri-urban and rural sites) (Table 1). In all cases, the bags were hung vertically from a short carbon fibre peg fixed perpendicularly to a pole at a height between 2 and 2.5 m, except in experiment 2.2.4 (see below). Thus, the moss bags were exposed in the field for periods of 4 weeks, except in experiment 2.2.3 (see below). During the different periods of the moss bag exposure, the main meteorological parameters (i.e. average temperature, wind direction and average bulk precipitation) managed by the regional meteorologic agency (www.meteogalicia.es) were recorded in the nearest automated meteorological stations to the studied SS (Table 2).

Three replicate moss bags were exposed in each SS. Some moss bags were not exposed in the field, to control for contamination during laboratory handling and transportation. As there were no differences between the final concentration of elements in these control moss bags and the initial concentration in unexposed moss, we con-

Table 2. Main climatological parameters recorded at the nearest automated monitoring stations present in the surrounding areas of the sampling sites (SS) during the different periods of exposure. Data provide by the regional meteorologic agency (www.meteogalicia.es) (AT: average temperature in ºC; WD: wind direction in º; P: Precipitation in L m^{-2}; n.a.= data not available).

					2010				2011
		March	April	May	Sept.	Oct.	Nov.	Dec.	January
SS1-4, 10	AT	n.a.	12.28	13.31	16.86	13.79	9.71	8.42	11.6
	WD	225	90	90	90	45	225	90	90
	P	71.7	48.6	85.2	36.2	185.9	214.9	127.3	134
SS5	AT	9.74	12.39	13.25	17.01	14.27	10.39	9.32	9.44
	WD	n.a.	45	45	45	45	225	180	45
	P	77.3	43.9	32.5	18.8	96.4	144.6	110.6	90
SS6	AT	n.a.	15.39	15.1	18.1	15.07	11.27	10.06	10.13
	WD	n.a.	90	45	45	180	45	180	45
	P	n.a.	n.a.	62.5	22.2	186	201.3	122.8	126
SS7	AT	6.15	9.11	9.98	15.17	12.16	7.93	6.69	6.83
	WD	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	P	65.1	31.9	71.9	26.3	122.7	171.1	246.72	151
SS8,9,13	AT	9.36	13.07	14.21	17.6	13.69	9.43	7.83	8.8
	WD	45	45	45	45	45	45	45	45
	P	134.7	70	84	31.4	212.8	180.1	228.4	227.4
SS11	AT	5.23	10.05	10.29	15.44	10.19	5.33	4.01	4.13
	WD	45	45	45	45	45	225	45	45
	P	142.6	37.7	81.2	28	237.3	223.57	122.5	180.8
SS12	AT	4.56	9.46	9.94	15.17	9.86	5.01	3.29	3.74
	WD	225	90	0	90	180	270	90	180
	P	165.3	67.2	101.5	18.7	418.2	220.3	210.23	315.8

clude that no contamination occurred.

2.2.1. Selection of species

Bags containing *S. denticulatum* and *P. purum* were exposed simultaneously at 5 SS: 4 SS were located in the surroundings of a steelworks at different distances from the factory (at distances between 213 and 820 m orientated in different directions from the main source) (Table 1) and 1 (control SS) was located in a rural area at a distance of 10 km from the factory. The experiment was carried out between September and December 2010, so there were in total 4 exposition periods.

2.2.2. Ratio between moss weight and bag surface area

Bags containing different weights (0.5, 1 and 4 g dry weight) of *S. denticulatum*, which provides different degree of bag coverage by the moss were exposed at 8 SS including rural, industrial and rural areas, to assess the optimal ratio between moss weight and surface area of the bag (Table 1). As the surface area of the flat bag was 176 cm^2, the resulting ratios were 2.84, 5.68, 22.72 mg cm^{-2}. The experiment was carried out twice, in March and September 2010.

2.2.3. Selection of duration of exposure

Three different durations of exposure (4, 8 and 12 weeks) were tested with bags containing *S. denticulatum*, in the same SS as the previous experiment (Table 1). The experiment was carried out twice, in March and September 2010.

2.2.4. Selection of height of exposure

Bags containing *S. denticulatum* were exposed at 8 different heights (i.e. 0.5, 1, 1.5, 2, 2.5, 3, 4, and 5 m) in 5 SS (Table 1) including industrial, urban and rural areas. The experiment was carried out twice, in December 2010 and January 2011.

2.3. Sample preparation and chemical analysis

At the end of the exposure period, the moss bags were dried at 40 ºC until constant weight. The moss tissue was then homogenized in an ultracentrifugal mill (Restch ZM 200, heavy metal-free).
Mercury was determined in an elemental analyzer (Milestone DMA 80) and Zn was determined by flame atomic absorption spectrometry (Perkin Elmer 2100); the concentrations of Cd, Cu and Pb were determined in solid suspensions by graphite furnace atomic absorption spectrophotometry (Perkin Elmer AAnalyst 600).
To control the analytical quality, analytical replicates were processed, 1 every 10 samples. The standard deviation was calculated as follows (Čeburnis and Steinnes, 2000):

$$S = \sqrt{\frac{\sum_{n}^{i} (C_1 - C_2)_i^2}{2n}}$$

Where n is the number of samples and C1 and C2 are the respective concentrations of duplicates. Certified reference material (M2 and M3, *Pleurozium schreberi*, Steinnes et al., 1997) was analysed in parallel 1 every 10 samples. Contamination during processing was controlled for by the use of analytical blanks (1 every 10 samples analysed). Recovery of elements from the reference materials was satisfactory and ranged between 75% for Hg and 125% for Pb. The overall error associated with the analytical process was usually lower than 5% and never higher than 10%. The concentrations of Cd, Cu, Hg, Pb and Zn obtained for each of the options and methodological aspects were always higher than the limit of quantification (LOQ) of the chemical analyses.

2.4 Data analysis

The limit of quantification of the technique (LOQ_T) was calculated from the initial concentrations as follows: $xC_i + 10sC_i$, where xC_i is the mean value of the initial concentration in unexposed moss for each element determined, and sC_i is the corresponding standard deviation (Couto et al., 2004). Calculation of the LOQ_T enables clear distinction of the concentrations in exposed and unexposed moss, which is also subject to different sources of variability. This should not be confused with the LOQ of the analytical process with analytical standards. Enrichment factors (EFs)

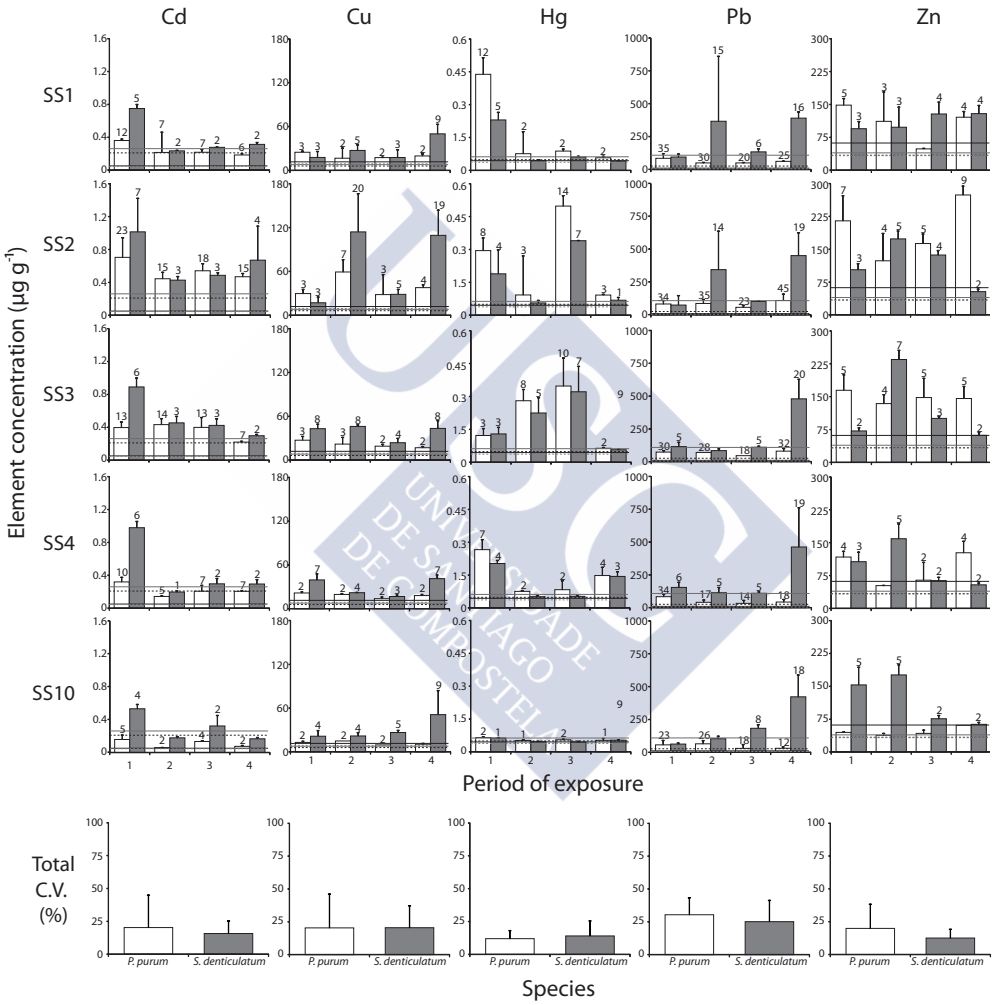


Fig. 1. Mean concentrations (µg g^{-1} dw) of Cd, Cu, Hg, Pb and Zn in *Pseudoscleropodium purum* (white bars) and *Sphagnum denticulatum* (grey bars) exposed at 5 sampling sites (SS) for four exposure periods, vertical lines shows the standard deviation. Initial concentrations are represented by dashed grey (*P. purum*) and black (*S. denticulatum*) lines. Limits of quantification of the technique (LOQ_T) are represented by solid black (*P. purum*) and grey (*S. denticulatum*) lines. Enrichment factors (EFs) are shown only for those mean concentration values higher than the LOQ_T. The final row of histograms shows the coefficient of variation (C.V.) for each of the species tested, n=3; total n=60.

were calculated as the ratio between the concentrations at the end of the exposure period and the initial concentrations (C_i). A Pareto Chart was constructed to help prioritize the methodological aspects for standardization (Cervone, 2009; Talib et al., 2010). This chart is a graphic representation of the degree of importance of the different factors or, in this case, the methodological aspects that affect a process or result.

3. Results

3.1. Differences in heavy metal concentration and replicability

The best option was selected on the basis of the number of cases in which final concentrations are higher than the LOQ_T and of the degree of replicability. The results for initial concentrations and LOQ_T respectivelyin *S. denticulatum* samples are: Cd (ng g^{-1}) 151.32 and 191.93; Cu (µg g^{-1}) 5.67 and 7.65; Hg (ng g^{-1}) 48.14 and 61,37; Pb (µg g^{-1}) 24.05 and 105.88; Zn (µg g^{-1}) 32.08 and 38.07. Regarding *P. purum* Cd (ng g^{-1}) (initial concentration and LOQ_T respectively) 30.54 and 36.67; Cu (µg g^{-1}) 8.65 and 11.69; Hg (ng g^{-1}) 36.14 and 42.98; Pb (µg g^{-1}) 2.47 and 3.66 and Zn (µg g^{-1}) 32.15 and 61.29 respectively for initial concentrations and LOQ_T.

3.1.1. Selection of species

Regarding the selection of species (Fig. 1), the tissue loads of all elements, except Zn, depended on the moss species used; this was found for all 5 SS and all 4 exposure periods. The concentrations of Cd, Cu and Pb were higher in *S. denticulatum* than in *P. purum*. For Cd, a contamination signal (i.e. concentrations>LOQ_T) was detected in both moss species, for all SS and exposure times; however, the replicability of the *S. denticulatum* data was higher. For Cu, a signal was detected in all SS and for all exposure times, except for one instance in *P. purum* (i.e. SS10 exposure period 4); the replicability was similar in both species, but as the EFs were higher in *S. denticulatum*, this species is recommended for monitoring Cu. By contrast, although in all cases the concentrations of Pb were higher in *S. denticulatum* than in *P. purum*, the EFs were lower because the LOQ_T was approximately 106 µg g^{-1}, whereas for *P. purum* the corresponding value was approximately 3 µg g^{-1}, and in 6 instances no contamination signal was detected in *S. denticulatum*. Therefore, although the data replicability was slightly higher in this species, *P. purum* was finally considered to be the best option for monitoring Pb. For Hg, a different pattern of uptake was detected, in which the final concentration, the signal detected and the replicability were higher in *P. purum* than in *S. denticulatum*. Finally, the results for Zn were more variable regarding the final concentration of the elements, although *S. denticulatum* was the best option for monitoring this element as no contamination signal was detected in *P. purum* in 5 instances and the variability was also higher in this species.

3.1.2. Ratio between moss weight and surface area of the mesh bag

For all elements, there was a general decrease in the final concentrations as the weight of moss increased (Fig. 2); this was constant for all exposure periods and was more evident in those sites in which the higher concentrations of contaminants were detected. For Cd, a contamination signal was detected in all cases, and therefore the best option was 22.72 mg cm^{-2}, following the criterion of replicability. For Cu and Zn, the best option was 5.68 mg cm^{-2} as this yielded the largest signal and the C.V. values were lower than with the other options. Finally, for Hg and Pb, the

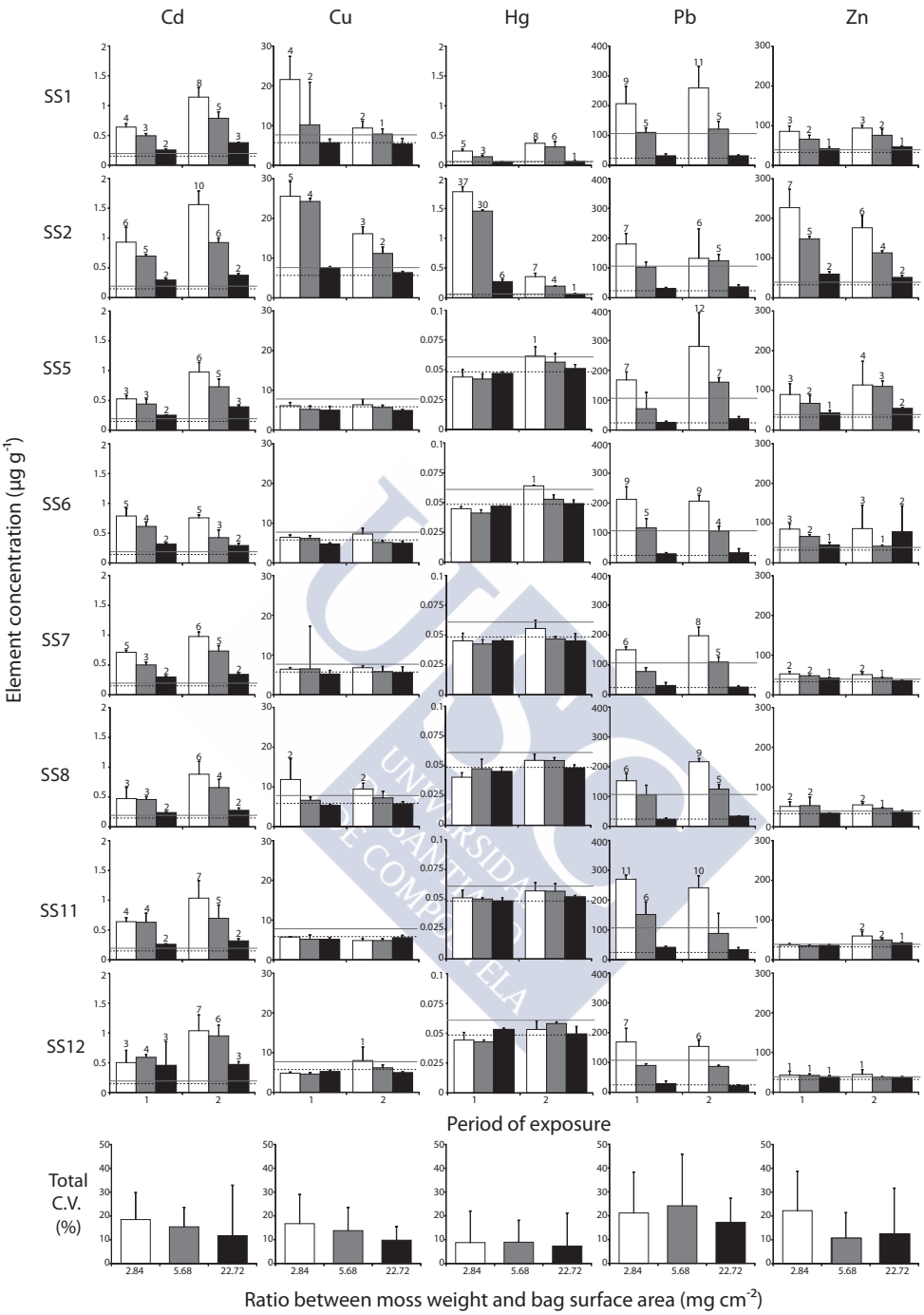


Fig. 2. Mean concentrations (µg g^{-1} dw) of Cd, Cu, Hg, Pb and Zn in relation to the quantity of moss weight per bag surface area, for *Sphagnum denticulatum* bags (white bars=2.84 mg cm^{-2}; grey bars= 5.68 mg cm^{-2}; black bars= 22.72 mg cm^{-2}) exposed at 8 sampling sites (SS) for two exposure times, vertical lines shows the standard deviation. Initial concentrations are represented by a dashed black line, and limits of quantification of the technique (LOQ_t) are represented by a solid grey line. Enrichment factors (EFs) are shown only for those mean concentration values higher than the LOQ_t. The final row of histograms shows the coefficients of variation (C.V.) for the relationships between moss weight and bag surface area tested, n=3; total n=30.

22.72 mg cm^{-2} option yielded the highest degree of replicability; however, the concentrations obtained were below the LOQ_T in all cases for Pb and in 13 instances for Hg, whereas with the 2.84 mg cm^{-2} option, the signal was more robust and less variable in all cases.

3.1.3. Selection of duration of exposure

The results for the uptake rate (μg g day^{-1}) in relation to duration of exposure indicate that it remains almost constant over time in all SS. The concentrations of heavy metals therefore increased with duration of exposure of the moss bags (Fig. 3). For Cd, the 4 week exposure period was the best option as no contamination signal was detected in most cases with the exception of two occasions (i.e. SS8 exposure period 2, SS11 exposure period 1) and the C.V. was the lowest of the three options tested. By contrast, the 8-week period was the best option for monitoring Cu, Pb and Zn, because a contamination signal was detected in most cases and also because the replicability data were the best for these elements and this option. In addition, in some cases the final concentrations of Pb and Zn were higher for the 8-week exposure period than for the 12-week exposure period. Finally, for Hg, although the variability was lowest in the 4-week exposure period, no contamination signal was detected in 9 instances, and therefore the optimal exposure period was 12 weeks; the final concentration was always higher than the LOQ_T and the data were not highly variable.

3.1.4. Selection of height of exposure.

Variable results were obtained for the different elements, SS and exposure period, therefore it was not possible to detect any vertical pattern of distribution (Fig. 4). However, in several instances (11 out of 50), the option that yielded the highest concentrations of all elements was a height of 5 m, although in the specific case of the busy roadside (SS9), the highest concentrations were found at a height of 0.5 m. However, the wide variability in the contamination signal for the different SS and exposure times precludes selection of one of the options as optimal on the basis of this requisite. For example, for Cd the variability was lowest at the exposure height of 1 m, whereas for Cu and Zn it was lowest at 2.5 m and for Hg and Pb at an exposure height of 4 m.

3.2. Pareto chart

To compare the overall C.V. for the different methodological aspects assessed, a Pareto chart was elaborated for each element determined (Fig 5). The height and duration of exposure introduced the highest degree of variability in the data set, for 3 (i.e. Cd, Pb and Zn) and 2 (i.e. Cu, Hg) metals respectively, whereas the moss species is associated with the lowest degree of variability in relation to the data set.

4. Discussion

During optimization of the moss bag technique, researchers should ensure that the different methodological aspects do not interact with each other. This can be done by an iterative optimization procedure in which the aspect under study is varied and all other aspects are held constant. However, this approach was not practicable here, as we wanted to evaluate 4 aspects simultaneously. We therefore worked under the assumption that there was no interaction between the study variables.

4.1. Concentrations of elements and data replicability

4.1.1. Selection of species

The species used in the moss bag tech-

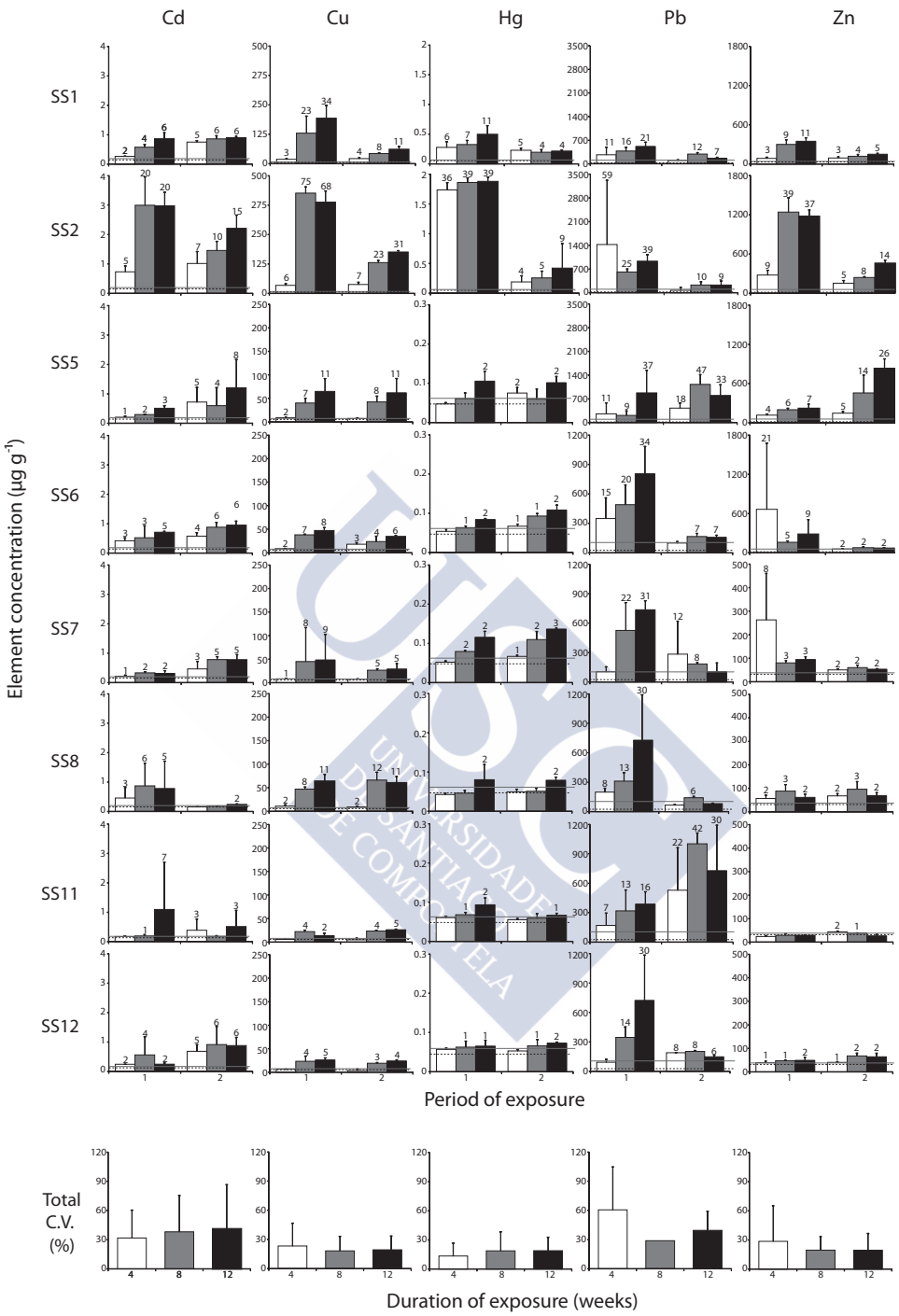


Fig. 3. Mean concentrations (µg g^{-1} dw) of Cd, Cu, Hg, Pb and Zn in relation to the duration of exposure using *Sphanum denticulatum* (white bars=4 weeks; grey bars= 8 weeks; black bars= 12 weeks) exposed at 8 sampling sites (SS) for two exposure periods, vertical lines shows the standard deviation. Initial concentrations are represented by a dashed black line, and limits of quantification of the technique (LOQ_T) are represented by a solid grey line. Enrichment factors (EFs) are shown only for those mean values higher than the LOQ_T. The final row of histograms shows the coefficient of variation (C.V.) for each exposure period tested, n=3; total n=30.

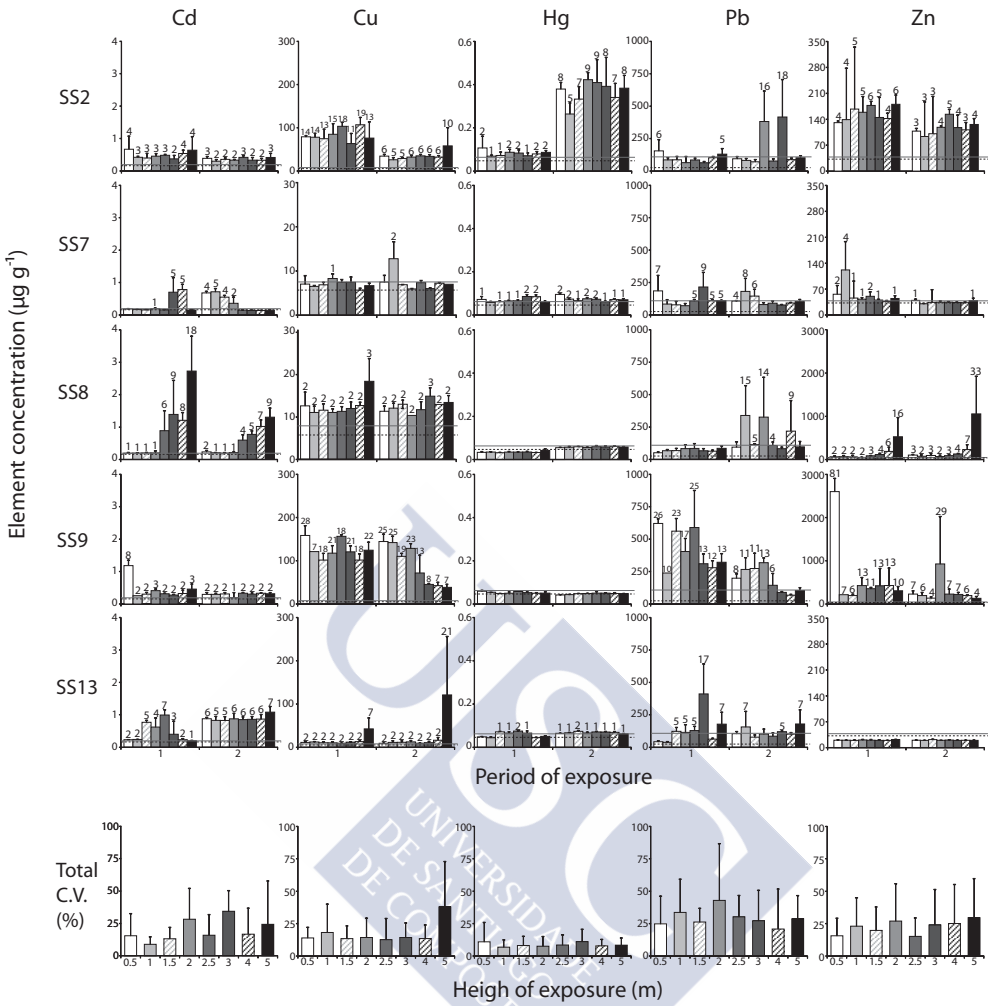


Fig. 4. Mean concentrations (µg g^{-1} dw) of Cd, Cu, Hg, Pb and Zn in relation to the duration of exposure using *S. denticulatum* (white bars=4 weeks; grey bars= 8 weeks; black bars= 12 weeks) exposed at 8 sampling sites (SS) for two exposure periods, vertical lines shows the standard deviation. Initial concentrations are represented by a dashed black line, and limits of quantification of the technique (LOQ_T) are represented by a solid grey line. Enrichment factors (EFs) are shown only for those mean values higher than the LOQ_T. The final row of histograms shows the coefficient of variation (C.V.) for each exposure period tested, n=3; total n=30.

nique greatly affects the results obtained. Of all the species usually used in this type of study, those of the genus *Sphagnum* have been found to accumulate the highest quantities of contaminants because of their morphological and physiological characteristics (Temple et al, 1981; Bargagli 1998; González and Pokrovsky 2014). Although no comparison has been made of the efficiency of element capture in *S. denticulatum* and *P. purum*, the capture efficiency of both of these species has been compared with that of *Hypnum cupressiforme* (Castello, 2007; Ratcliffe, 1975; Culicov and Yurukova, 2006), with the latter displaying the lowest accumulative capacity in all cases.

In the present study, we found that *S. denticulatum* was more suitable than *P. purum* for monitoring 3 of the 5 elements determined. Species of the genus *Sphagnum* are generally more appropriate for

this purpose because of their morphology. Thus, particle retention is enhanced by the large surface area, (determined by the number of phyllids per caulid; Bargagli, 1998), which is higher in *Sphagnum spp*. than in *P. purum*. Moreover, González and Pokrovsky (2014) have demonstrated that the element adsorption capacity depends on the concentration and type of functional groups present in the moss cell wall and that *Sphagnum spp*. possess a higher capacity to adsorb metals than other moss species due to the higher concentration of carboxilic groups in the cell wall.

Finally, the results for contamination signal and data replicability indicate that *P. purum* is suitable for monitoring Hg. This difference with respect to the other elements may be due, on the one hand, to a greater affinity for -SH groups (Pérez-Llamazares et al. 2009) and in this case, the number of sulfhydryl/amine/polyphenol binding sites is greater in *P. purum* than in *Sphagnum spp.* (González and Pokrovsky, pers. comm.).

With the exception of Hg, the results generally indicate that *S. denticulatum* is more suitable than *P. purum* for use in the moss bag biomonitoring technique.

4.1.2. Ratio between moss weight and bag surface area

The lack of a contamination signal for a moss weight /bag surface ratio of 22.72 mg cm^{-2} may be because particle interception was not maximal, unlike in the 2.84 mg cm^{-2} option in which the moss was placed in a thin layer and all of the shoots in the bag were equally exposed, with no overlapping or flattening (Zechmeister et al., 2006). For some elements, such as Cu, Hg and Zn, the final concentrations for

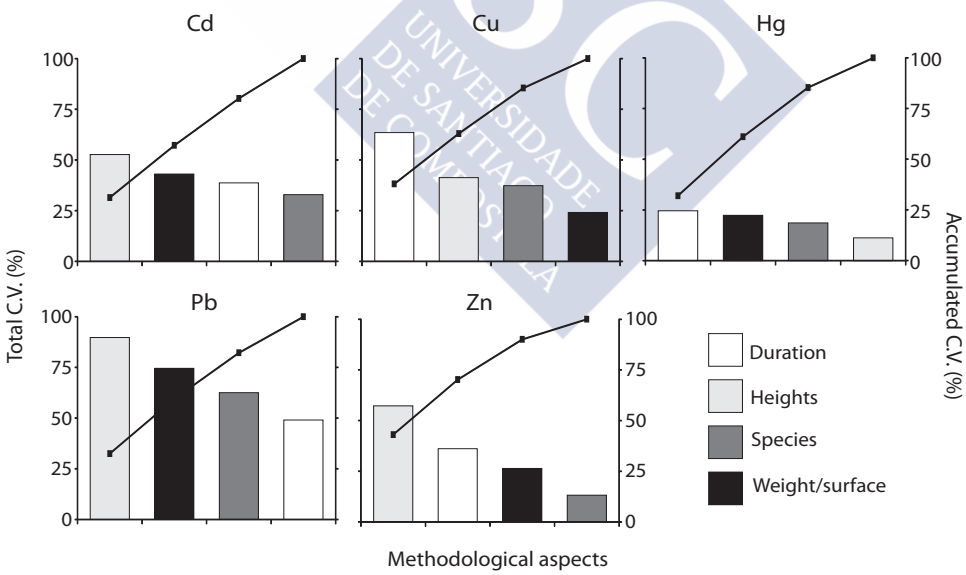


Fig. 4. Mean concentrations (µg g^{-1} dw) of Cd, Cu, Hg, Pb and Zn in relation to the duration of exposure using *Sphagnum denticulatum* (white bars=4 weeks; grey bars= 8 weeks; black bars= 12 weeks) exposed at 8 sampling sites (SS) for two exposure periods, vertical lines shows the standard deviation. Initial concentrations are represented by a dashed black line, and limits of quantification of the technique (LOQ_T) are represented by a solid grey line. Enrichment factors (EFs) are shown only for those mean values higher than the LOQ_T. The final row of histograms shows the coefficient of variation (C.V.) for each exposure period tested.

all three exposure times are very similar to the initial concentrations and no general pattern of decreasing concentration was observed (e.g. Hg SS5-SS12 time 1), and therefore it can be assumed that there was no enrichment in these cases. The replicability data show the opposite pattern, and for Cd, Cu, Hg and Pb the lowest C.V. values detected were for the 22.72 mg cm^{-2} option. This may be due to differences in the arrangement of the moss within the bag for the different weights of moss tested. Thus, whereas in the 2.84 mg cm^{-2} option all of the shoots are separate within the bag, in the 22.72 mg cm^{-2} option, the shoots overlap and most of the moss is not exposed to the atmosphere at the surface of the bag. The lower weight of moss is therefore more susceptible to particle deposition. On the other hand, the concentrations of elements are more homogeneous for the greatest weight of moss, as most of the moss has not been exposed to the atmosphere, because most of the shoots are completely compressed, so element concentration is more similar to the intial concentrations.

Taking all of the above into account, we propose an intermediate moss weight/bag surface surface ratio of 5.68 mg cm^{-2} as a compromise between contamination signal and data replicability.

4.1.3. Duration of exposure

Although the concentrations obtained for the three exposure periods generally increased over time, there were some exceptions indicating that this trend is not equally robust in all cases (e.g. Pb in exposure period 2 and Zn in SS6 and SS7 during exposure period 1). Thus, the moss is not integrating the contamination and the final concentrations reflect the balance between inputs and loss due to diverse factors. Such factors may include differences in the atmospheric levels of contaminants during different exposure periods, weather conditions that may affect the pollutant availability and the impact of airborne particles and their interception (Aničić et al., 2009a). However, the results for the rate of uptake for the three exposure periods show that this does not depend on the duration of exposure, which tends to be temporally stable. Previous studies have shown that the greatest changes in the rate of uptake are produced in shorter exposure periods (i.e. 1 and 2 weeks; Vasconcelos and Tavares, 1998; Aničić et al., 2009b).

Finally, the data replicability tended to improve with the time of exposure of the transplants; the data obtained in the 4-week exposure was more variable than the data obtained in the other periods. Indeed, he C.V. was highest during this period for 3 of the 5 elements determined. A similar trend was observed in previous studies in which the highest C.V. values corresponded to the shortest exposure times; the values then decreased after 30-45 days, especially for elements such as Hg, Pb and Zn (Galey and Lloyd, 1986b). Therefore, having into account the results for the previous and the present study (in terms of signal concentration and replicability), exposure of the moss bags for a period of 8 weeks fulfilled the established criteria.

4.1.4. Height of exposure

The lack of any vertical patterns of concentration and the high degree of variability in the results precludes selection of a single height at which the contaminant load will be higher for all elements and that will yield a highly replicable contamination signal. At SS9, which represents the area beside a busy road, no vertical pattern of contamination was detected. However, the concentrations of the elements determined were higher at an exposure height

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Table 3. Modification of the recommendations made by Ares et al. (2012)

Variable	New recommendations
1. Preparation of the moss	
1.1. Selection of species	*Sphagnum sp*[e] preferably from a cloned culture.
1.2. Selection of material	Green apical portions (5 cm)
1.3. Pre-exposure treatments and vital state	Devitalized
1.3.1. Washing with cellular extractants	
1.3.1.1. Number of washes	1 wash with EDTA (10 mM) + 1 with Dimercaprol (30 mM)
1.3.1.2. Duration of washing	20 minutes
1.3.1.3. Shaking	Wash with shaking
1.3.1.4. Weight of moss per volume of extractant	1 L water per 12 g dw
1.3.2. Washing with water	Water washed
1.3.2.1. Number of washes	3 times
1.3.2.2. Duration of washing	20 minutes
1.3.2.3. Shaking	Wash with shaking
1.3.2.4. Type of water	Distilled water
1.3.2.5. Weight of moss per volume of water	10 L water per 100 g dw
1.3.3. Devitalizing treatment	Oven drying, 24 h 120ºC[a,b]
2. Preparation of transplants	
2.1. Mesh material	Nylon mesh
2.2. Mesh size	2 mm[d]
2.3. Shape of bag	Spherical[c] (ensuring single layer of moss)
2.4. Size of bag	12 mg cm^{-2} [e]
3. Exposure	
3.1. Shading system	No conclusion
3.2. Cover	No conclusion
3.3. Location and type of support used	Free of obstacles, suspended from inert support by nylon thread
3.4. Height of exposure	4 m[e]
3.5. Duration of exposure	8 weeks[e]
3.6. Number of bags for site	3 bags
3.7. Initial concentrations and controls	3 controls and 3 initial times
4. Post exposure treatment	No treatment

[a] Fernández et al. (2010); [b] Giordano et al. (2009); [c] Gailey and Lloyd (1986c); [d] Giordano et al. (2013); [e] present study

of 0.5 m in 5 of the 10 cases. This may be due to the specific location of the sources of emission, i.e. the vehicle exhaust pipes (fumes) and wear and tear of wheels and asphalt (particles). The high variability in the results is due to the complex process of dispersion of contaminants in air masses and surface deposition, which is affected by factors related to atmospheric conditions or the type of distribution of contaminants (i.e. association with particles of different sizes or in gas phase), as well as aspects such as air turbulence, source of emission or topography of the area (i.e. presence of walls, buildings). Previous studies in which the influence of the height of exposure on element capture has been examined have yielded very different results, even though all were carried out in street canyons. Thus, De Nicola et al. (2013) concluded that there were no differences between *H. cupressiforme* transplants exposed at heights of 3, 6 and 9 m in Naples. In the same city, Adamo et al. (2011) found that *H. cupressiforme* bags exposed at a height of 4 m were more efficient at retaining contaminants associated with traffic and suspended dust

than samples exposed at a height of 20 m, which captured contaminants associated with long distance transport and cations of marine origin. Rivera et al. (2011) also did not find any differences between *H. splendens* bags exposed on balconies at heights of between 3 and 21 m in Girona (NE Spain). However, in a study carried out in Belgrade with *Sphagnum girgensohnii*, Vukovic et al. (2013) observed higher concentrations of elements at a lower exposure height (i.e. 4m) than at higher heights (i.e. 8 and 12 m).

Although the results were highly variable and different contamination processes may be captured at different heights, a specific height must be established to standardize this aspect; exposure heights of 2.5 m and 4 m yielded the most replicable results. Taking into account practical considerations (e.g. to avoid vandalism) (Ares et al., 2012), an exposure height of 4 m is recommended; however, different exposure heights can be used depending on the particular study objectives.

4.2. Pareto chart

Results show that the selection of the height of exposure accounts for most of the variation in the Cd, Pb and Zn data, and the duration of exposure explains most of the variation in the Cu and Hg data. The least important methodological aspect regarding the standardization process is the selection of species. The Pareto chart (Fig. 5) shows that the Pb data was the most variable and the Hg data was the least variable. This may be related to the different form in which Hg is present in the atmosphere (i.e. 95-99% in gas phase) relative to the other elements so can be more stably bounded to the –SH groups. Indeed Lodenius et al. (2003), demonstrated that Hg shows strong adsorption to *Sphagnum capillifolium* tissue, so the dynamics of deposition seems to be different from those of other elements which tends to adhere to particles of different sizes. Particle deposition may generate a greater degree of heterogeneity in the different replicates.

5. Conclusions

Traditionally, selection of one or other option as regards the methodological aspects of the moss bag technique has been done on the basis of maximal capture of elements. However, in the present study, the best options were determined on the basis of the contamination signal and the degree of replicability, the selection of species, the quantity of moss per surface area of the bag, duration of exposure and height of exposure. Moreover, in each case, the choice of the best option was made by taking into consideration sites affected by different levels of contamination and at least 2 exposure periods at different seasons during a year for 5 of the heavy metals most commonly determined in the moss bag biomonitoring technique.

On applying the technique, the height and duration of exposure are the aspects that introduce the most variability in the results, at least for the elements determined here. Use of the established protocol is therefore particularly recommended in regard to these aspects in order to minimize the variability.

The aim of this methodological study was to achieve further progress in the standardization of the moss bag technique. The results obtained have enabled us to improve on the preliminary protocol proposed in a previous study (Ares et al., 2012) (Table 3). However, more research is still required with the aim of further standardizing the technique for use as a regular environmental monitoring tool by official organizations.

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Chapter III



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Do moss bags containing devitalized *Sphagnum denticulatum* reflect heavy metal concentrations in bulk deposition?

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Abstract

Trace elements in atmospheric deposition have always been an environmental and human health concern. The most common approach to monitor their concentration in bulk deposition is through the use of traditional methods although the use of the moss bag technique entails a simpler and economical complementary technique. However some theoretical aspects of the latter technique are not still understood, such us the relationship between the levels of heavy metals accumulated in moss and the concentrations of these in the atmosphere. In the present study, the correlation between trace element concentration (i.e. Cd, Cu, Hg, Pb and Zn) in devitalized moss bags using *Sphagnum denticulatum* and bulk deposition was studied, during 12 periods of exposure in 21 sampling sites (SS) affected by different degrees of contamination. Most of the significant correlations involved Cd and to a lesser extent Cu and Zn, however no significant correlations were found for Pb and Hg. The environmental conditions and particularly the abundance of precipitation are the main causes of the absence of correlations in some cases, presumably because of the loss of elements by leaching. However, despite the limits of the method must always be borne in mind, the moss bag technique is a very useful and economical environmental tool

Keywords: heavy metals, active biomonitoring, environmental tool, contamination.

1. Introduction

The presence of trace elements in atmosphere has long been of concern in relation to environmental and human health. Several measures have been taken to control the atmospheric deposition of trace elements at the European level. Thus, EU Directive 1999/30/EC requires to the European Union member states the measurement of Pb, and the EU 4th Daughter Directive 2004/107/EC also requires measurements of atmospheric deposition of Cd, As and Ni. Furthermore, parties to the Convention on Long-range Transboundary Air Pollution (CLTRAP) are obliged to monitor several trace elements (Pb, Cd, Hg, Cd, Pb, Cu, Zn, As, Cr and Ni).

Also, the US Environmental Protection Agency (USEPA) sets limits on certain air pollutants, including trace elements such as Pb and Hg, by the implementation of different laws and regulations (e.g. the 1990 Clean Air Act). Thus, measurement of deposition rates and concentrations of these contaminants has been an object of study in the last few decades, and several protocols regarding the way in which these pollutants must be determined have been established (e. g. Aas et al., 2009, Alonso-Rodríguez et al., 2007). The most common approach in measuring trace elements in the total atmospheric deposition is through the use of traditional or physicochemical methods. At present, the standard method used to determine As, Cd, Pb and Ni in atmospheric deposition is that established by the European Committee for Standardization (EN15841:2009).

In addition to traditional methods, a simple, economic, active biomonitoring method with terrestrial moss, known as the moss bag technique, can be used in to estimate air quality. This method provides particular advantages for the study of industrial and urban environments where the presence of native moss is limited or absent. However, although more than 120 scientific articles on the use of the moss bag technique have been published, the method cannot be validated for routine application by public authorities as the methodological and theoretical bases for the technique have not yet been completely established. In one hand, in recent years several studies have addressed various methodological aspects (e.g. Ares et al., 2012; Ares et al., 2014; Fernández et al., 2009; Giordano et al., 2009; Tretiach et al., 2007), with the aim of standardizing the protocols for preparing and exposing the moss bags and thus enhancing the quality of data obtained and enabling interpretation of the results. On the other hand, regarding the theoretial aspects, the relationship between the levels of heavy metals accumulated in moss and the concentrations of these in the atmosphere are still not well understood. It is known that capture of elements by moss is mainly due to passive uptake of particulate matter (e.g. Aničić et al., 2009; Basile et al., 2009; Giordano et al., 2009), and recent comparisons between the trace element content in mosses and in airborne particle matter (especially PM_{10}) (e.g. Adamo et al., 2007; De Nicola et al., 2013; Tavares and Vasconcelos, 1996; Vuković et al., 2014) have shown that moss may intercept up to 98% of PM_{10} particles (Tretiach *et al*., 2011). However, the significance of the results in terms of metal concentrations in bulk deposition has rarely been discussed, and only 3 studies have considered the correlation between concentrations of heavy metals in moss bags and in bulk deposition: two of these studies were carried out in urban environments and used irrigated and dry *Sphagnum* spp. (Aničić et al., 2009; Duggan and Burton, 1983) and the other study was carried out in the surroundings of a steelworks (Pilegaard, 1979) and used *Dicranoweisia cirrata*. However, the levels of Hg were not determined in any of these studies, despite the fact that monitoring of this element is required under the prevailing legislation.

Numerous studies have compared the concentrations of elements in bulk deposition and native moss, and it has been concluded that the moss does not always accurately reflect the atmospheric levels of certain elements. This may be due to the physicochemical characteristics of pollutants and the physicochemical processes in mosses (Aboal et al., 2010). Regarding the former, Varela et al. (2014, submitted for publication) found that certain characteristics such as the ionic radius and the covalent bond index have important effects on

the uptake of elements from the atmosphere. In the present study, we determined the concentrations of Cd, Cu, Hg, Pb and Zn in moss bags and bulk deposition, as the concentrations of these elements in the atmosphere and moss are generally well correlated becuase of the characteristics mentioned above. Moreover, monitoring of Cd, Hg and Pb is contemplated in current legislation.

Validation of the method requires calculation of the variability in the results in urban, industrial and rural environments, although the sampling site can be assumed to be an important source of variability (Tretriach et al., 2011). The effects of the different seasons on the results should also be considered.

Taking into account all the information mentioned above, the main aim of the present study was to examine the relationship between the concentrations of Cd, Cu, Hg, Pb and Zn measured in moss bags and in the bulk deposition in different environments and at different times of year. For this purpose, we tested the null hypothesis of no correlation between the concentrations in the bulk deposition and moss, independently of sampling site or time of year.

2. Material and methods

2.1 Preparation of moss and transplants

The details regarding preparation of the moss bags are described in a previous study (Ares et al., 2014). Briefly, moss bags were prepared with the green parts of *Sphagnum denticulatum* Brid. The moss was distributed homogeneously inside flat bags (2.5 mg of moss cm^{-2}), minimizing overlapping and compression of the moss. The moss was then devitalized by drying in an oven at 120° C for 24 h (Giordano et al., 2009). Finally, the bags were vacuum packed and stored at -20° C until use.

2.2. Study area and experimental set-up

The study was carried out in Galicia (NW Spain) (Fig 1). In total, 21 sampling sites (SS) affected by different degrees of contamination were selected for study. The SS included different industrial environments (woodworks: SS1, coal fired power plant: SS2-SS6, steelworks: SS7-SS11, and an alumina-aluminium smelter: SS12-SS14), peri-urban (SS15-SS17) and unpolluted areas (SS18-SS21). The prevailing weather conditions (i.e. average temperature, wind direction and total precipitation) were recorded monthly at the meteorological monitoring stations closest to the SS (data provided by the regional meteorological agency (Meteogalicia-Xunta de Galicia; www.meteogalicia.es) (Table 1).

Three replicate bulk deposition collectors and three replicate moss bags were installed in each SS. The bulk deposition collector consisted of a combination of funnel (22 cm diameter) and bottle. Each funnel was covered with a plastic net (0.5 cm mesh size) to prevent entry of plant and animal remains. The collecting surfaces (collector and funnel) were made of non-metal containing material (i.e. high density polyethylene), and the containers were opaque, to prevent growth of algae. 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).

One moss bag was hung from a short carbon fibre peg fixed perpendicularly to a pole at a height of ca. 2.5 m over each bulk deposition collector.

Samples of bulk deposition and moss bags were collected every 4 weeks for 1 year, be-

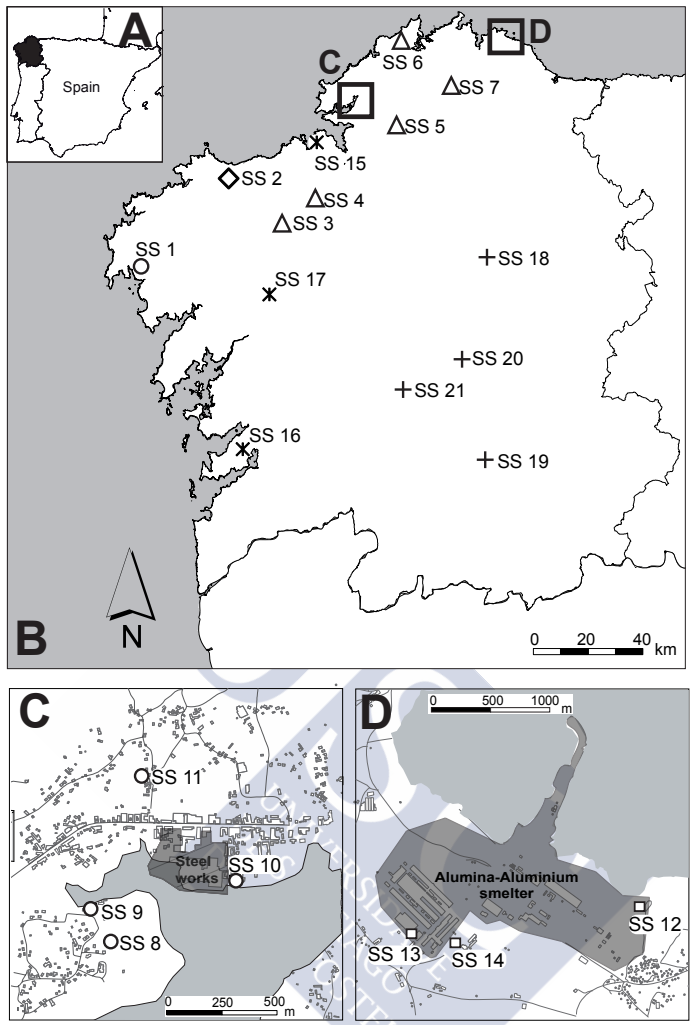


Fig. 1 A. Map showing the location of Galicia in Spain (in black). B map showing the geographical location of the 21 sampling sites (SS), and C and D are enlargements showing the locations of the steelworks and alumina-aluminium smelter respectively. Rhombus= woodworks, triangle = coal fired power plant, circle= steel works, squares=alumina-aluminium smelter, asterisk=peri-urban areas and cross= rural areas.

tween January and December 2010.

2.3. Collection of samples

Samples of bulk precipitation were collected after washing the funnel with 100 mL of bidistilled water to remove any particles adhered to the surface of the funnel, and the contents of the bottle were shaken. The total volume of precipitation in each collector was recorded and an aliquot of the bulk deposition was collected in a Falcon tube (15 mL). All samples were acidified with hyperpure HNO_3 (added to a final concentration of 1%) and stored at -20ºC until chemical analysis.

Some moss bags were not exposed in the field, to control for contamination during laboratory handling and transportation. As there were no differences between the final concentration of elements in the control moss bags and the initial concentration in unexposed moss, we concluded

that no contamination occurred during laboratory handling and transportation of the moss bags.

2.3. Sample preparation and chemical analysis

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. The recoveries ranged between 67% for Cu to 108% for Cd. After filtration of the samples through a 45µm filter, Hg was determined by ICP-MS (NexION® 300X, PerkinElmer Inc., Shelton,CT), at the Department of Analytical Chemistry, Nutrition and Bromatology, University of Santiago de Compostela. Recovery of the certified reference material (Environment Canada Total Mercury PT97-Hg07) was around 103%. The concentrations of Cu, Pb and Zn in the bulk deposition samples were above the quantification limits (LOQ), whereas those of Cd and Hg were below the quantification limits in 9% and 56% of analyses respectively.

After exposure of the moss samples in the field, the bags were transported to the laboratory where the moss was homogenized in an ultracentrifuge mill (Restch ZM 200, heavy metal-free). The samples were then dried at 40 ºC until constant weight. The details of the chemical analysis of the samples are outlined in a previous study (Ares et al., 2014). Briefly, Hg was determined in an elemental analyzer (Milestone DMA 80) and the concentrations of Zn Cd, Cu and Pb were determined in solid suspensions by graphite furnace atomic absorption spectrophotometry (Perkin Elmer AAnalyst 600). Zinc was determined by flame atomic absorption spectrometry (Perkin Elmer 2100) (Barciela et al., 2014, submitted for publication).

All quality control samples (i.e. analytical replicates, certified reference material and analytical blanks) were analyzed once every 10 samples. Recovery of elements from the reference materials (M2 and M3, *Pleurozium schreberi*, Steinnes et al., 1997) was satisfactory and ranged between 70% for Hg and 131% for Pb. The overall error associated with the analytical process was usually lower than 5% and never higher than 10%. All determinations were above the corresponding LOQ.

2.4. Data Analysis

The limit of quantification of the technique (LOQ_T) was calculated from the initial concentrations, as follows: $LOQ_T = xCi + 1.96sCi$, where xCi is the mean value of the initial concentration in unexposed moss for each element determined, and sCi is the corresponding standard deviation (Couto et al., 2004). In the present study, we lowered the limit established in the afore mentioned study from 10sCi to 1.96 sCi, as if we assume that the concentrations are normally distributed at the start of the exposure period (initial concentrations), inclusion of the new value in the formula ensures that the probability of the data belonging to the distribution at initial concentrations will be less than 2.5%. The LOQ_T should not be confused with the LOQ of the analytical process with analytical blanks.

The standard uncertainty was estimated using independent field replicates in order to calculate the between-replicate uncertainty, for bulk deposition and moss bags samples, in each of the SS studied. This parameter was calculated according to the European Commission's working group on Guidance to Equivalence Demonstration (2007) and the International Standards Organisation (2007), as follows (Aas et al., 2009):

$$u(y) = \sqrt{\frac{\sum_{j=i}^{N} S(j)^2}{N}}$$

where u(y) is the standard uncertainty, N is the number of field replicates, and S(j) is the standard deviation of the concentration in each SS.

Once the mean concentration grouped by SS and period of exposure of contaminants in moss and bulk deposition was calculated, the Shapiro–Wilks test (n=12 for SS and n=21 for the period of exposure) was used to check the normality of the distribution of the concentrations. As neither the raw data nor transformed data were normally distributed, a non parametric Spearman's correlation test was used to establish any relationship between the mean concentration of contaminants in the bulk deposition and moss samples grouped by sampling site (SS) data and by period of exposure. IBM SPSS, version 20.0, was used to carry out all statistical tests.

Table 1. Main climatological parameters recorded at the nearest automated monitoring stations present in the surrounding areas of the sampling sites (SS) during the different periods of exposure. (AT: average temperature in ºC; WD: wind direction in º; P: precipitation in L m^{-2}). Data provide by Meteogalicia.

	Month	Jan.	Feb.	March	April	May	June	July	August	Sept.	Oct.	Nov.	Dec.
SS1	**AT**	9	9	10	12	14	16	17	18	17	15	11	10
	WD	45	225	90	90	45	45	45	45	45	225	270	90
	P	159	114	80	33	66	38	9	8	28	101	136	96
SS2	**AT**	8	8	10	13	14	17	19	19	17	14	10	8
	WD	180	180	180	45	315	315	315	315	315	180	180	180
	P	184	160	84	45	85	117	38	12	39	252	247	123
SS3	**AT**	5	5	7	11	12	14	16	16	16	12	8	6
	WD	45	0	225	0	45	45	45	45	45	180	225	180
	P	262	290	156	58	109	95	39	21	32	285	286	172
SS4	**AT**	4	4	6	10	11	14	16	17	15	11	7	5
	WD	45	225	90	90	0	45	90	45	45	90	225	135
	P	182	220	128	81	82	139	24	14	33	276	300	149
SS5	**AT**	8	8	10	12	13	15	17	18	17	14	10	9
	WD	45	-	-	45	45	45	45	45	45	45	225	180
	P	130	84	77	44	33	91	32	19	19	96	145	111
SS6	**AT**	-	-	-	-	15	16	19	19	17	14	10	8
	WD	-	-	-	-	-	90	90	90	90	225	225	225
	P	-	-	-	-	66	222	18	16	21	218	279	268
SS7	**AT**	-	-	-	15	15	17	20	20	18	15	11	10
	WD	-	-	-	90	45	45	45	45	45	180	45	180
	P	-	-	-	-	63	65	17	14	22	186	201	123
SS8,9,10,11	**AT**	8	7	9	12	13	16	18	18	17	14	10	8
	WD	45	225	225	90	90	45	45	45	90	45	225	90
	P	215	128	72	49	85	144	50	25	36	186	215	127
SS12,13,14	**AT**	5	4	6	9	10	13	15	16	15	12	8	7
	WD	-	-	-	-	-	-	-	-	-	-	-	-
	P	213	125	65	32	72	218	32	23	26	123	171	247
SS15	**AT**	10	10	11	13	14	17	18	19	17	15	12	10
	WD	180	180	180	180	0	0	0	0	180	180	180	180
	P	103	79	33	24	50	54	20	3	19	116	143	92
SS16	**AT**	9	9	11	14	15	19	21	22	18	15	11	9
	WD	135	90	270	270	315	315	135	135	315	270	270	90
	P	197	232	147	49	113	65	24	3	41	255	210	196
SS17	**AT**	8	7	9	13	14	17	19	20	18	14	9	8
	WD	45	45	45	45	45	45	45	45	45	45	45	45
	P	200	306	135	70	84	56	27	10	31	213	180	228
SS18	**AT**	6	6	8	12	13	16	19	19	16	12	8	5
	WD	180	135	135	0	0	0	0	0	0	180	180	180
	P	117	169	89	60	34	124	27	4	24	196	187	150
SS19	**AT**	5	5	8	11	12	16	18	19	16	12	7	5
	WD	-	-	-	-	-	-	-	-	-	-	-	-
	P	105	180	98	72	62	76	28	3	20	151	136	133
SS20	**AT**	2	2	5	9	10	14	18	18	15	10	5	3
	WD	180	225	225	90	0	0	90	90	90	180	270	90
	P	246	275	165	67	102	91	11	14	19	418	220	210

3. Results

At the end of the exposure period, the concentrations of most of the elements in the moss samples were higher than the LOQ_T (Fig. 2 and 3), with exception of Cd (in 14% of the cases), Cu (6%), Hg (30%) and Zn (1%).

The standard uncertainty was calculated for field replicates of moss and bulk deposition samples in each SS. Average data was calculated for each SS and exposure period (in total, 21 SS and 12 exposure periods). For Hg, having into account that most of the data were bellow the detection limits, standard uncertainty values were only calculated for moss bag samples. For moss bags, the lowest uncertainty values were associated with Hg and ranged between 5 and 8%. Comparisons revealed higher uncertainty values for bulk deposition than for moss bags. Thus, the overall standard uncertainty values for the different samplers were as follows: Cd, 10% and 28%; Cu, 12% and 22%; Pb, 21% and 24%; and Zn, 14% and 24% (moss bags and bulk deposition respectively).

Spearman's rank correlation coefficients (rho) were calculated in order to determine any relationships between trace element concentrations in moss and bulk deposition. Grouping the data on the basis of sampling sites yielded only two positive and significant correlations ($p<0.05$) for two industrial sites in the surroundings of a coal-fired power plant (SS3) and a steelworks (SS11). Grouping the data according to times of exposure revealed high positive correlations for Cd in April, July, August, September ($p<0.05$), in January ($p<0.01$), and in March and May ($p<0.001$). Significant correlations were also observed for Cu in January, April and June ($p<0.05$) and finally in May ($p<0.001$). Significant correlations were also found for Zn in June and August ($p<0.05$) and in May ($p<0.01$). However, no positive correlations were observed for Hg and Pb. Data showing significant correlations among trace element concentration in moss bags and bulk deposition are represented in Fig. 2, (grouped by SS), and in Fig. 3, (grouped by exposure period). In the latter, more significant relationships were detected in drier exposure periods, and the highest concentrations were related to industrial or peri-urban environments (see e.g. Cd: April, May, September), whereas contaminant levels were lower in both moss and bulk deposition in rural areas.

Figures 2 and 3 also include the straight line of maximum slope, which indicates the maximum concentration accumulated in the moss tissues for a given concentration in the bulk deposition at a particular sampling site (Fig. 2) or exposure period (Fig. 3). We assume that if there is no loss of elements, all of the points should be situated along the straight line. The point at which this line crosses the y axis represents the concentration of heavy metals at the start of the measurement period. In 12 of the 14 significant correlations, the maximum ratio of the concentration of elements in the moss to the concentration in the bulk deposition corresponds to industrial environments, and most of these (9 instances) correspond to the surroundings of a steelworks smelter. The slopes of the lines for all data were variable, especially for Cd (47-66000), Hg (49-257244) and Pb (332-161937). For Cu, the values of the slope range between 341 and 1770, for for Zn, they range between 137 and 2054.

Spearman's correlation test was also used to asses the possible influence of precipitation on element leaching. For this purpose, the residual values, i.e. estimated concentrations in moss (calculated from the equation for maximum slope) minus the observed concentration, were correlated with the amount of precipitation re-

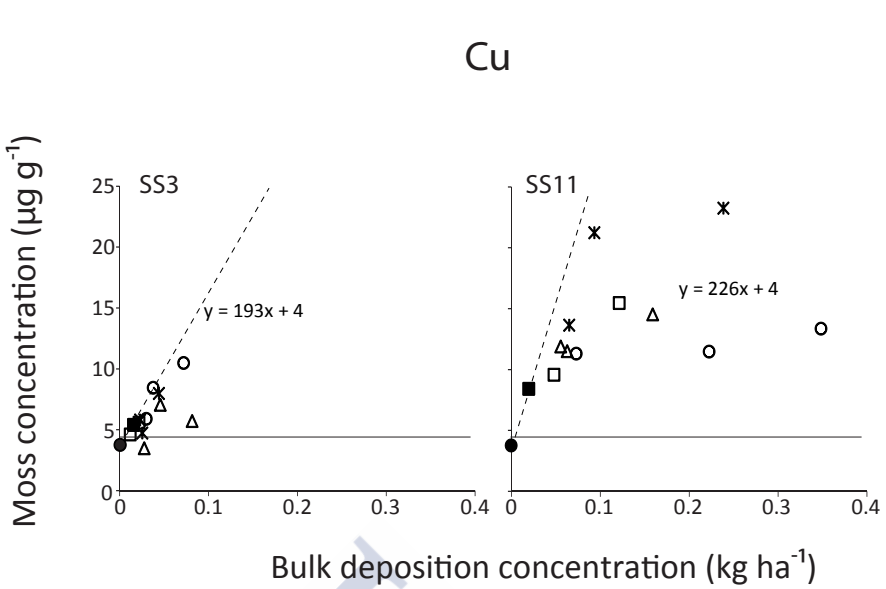


Fig. 2. Graphical representation of the concentration of Cd, Cu and Zn in *Sphagnum denticulatum* in relation to bulk deposition grouped by periods of exposure. Limits of quantification of the technique (LOQ_T) are represented by a solid black line, whereas the straight line of maximum slope is shown as a black dashed line. The equation for the slope is included in the graph. Rhombus= woodworks, triangle = coal fired power plant, circle= steel works, squares=alumina-aluminium smelter, asterisk=peri-urban areas and cross= rural areas.

corded at the meteorological stations closest to the SS studied for the data grouped by SS and exposure times. The residual values for Hg were also correlated with the mean temperature for the data grouped by SS and exposure time. This indicated the possible influence of temperature on the adsorption of Hg. Significant and positive correlations were only found between the residuals and the precipitation recorded for the data ordered by exposure time for Cd (in July and August), Cu (in June) and Pb (in January, June and August). No significant correlations were observed between the residual values for Hg and the mean temperature.

4. Discussion

In using the moss bag technique, it is essential to show that a signal has been obtained in the moss bags and that the level of variability in the data is acceptable, in order to confirm that the experimental design is appropriate for achieving the proposed objectives. Firstly, regarding the signal, in a study with devitalized *Sphagnum denticulatum*, we concluded that a period of 4 weeks was not sufficient to detect concentrations higher than the LOQ_T and that this duration of exposure also generated more variability in the results than longer exposure periods (Ares et al., 2014). However, in the previous study, a stricter limit was established for calculating the LOQ_T (10sCi instead of 1.96sCi). In the present study, no signal was detected for some elements, especially Cd and Hg. This either indicates that there was no contamination by these elements in the study area, or that an exposure time of 4 weeks was not sufficient to detect a signal in the concentration that differentiated it from the concentration at the start of the studied period. An exposure period of 4 weeks was chosen because studies carried out to determine the concentrations of contaminants in bulk deposition use monthly or

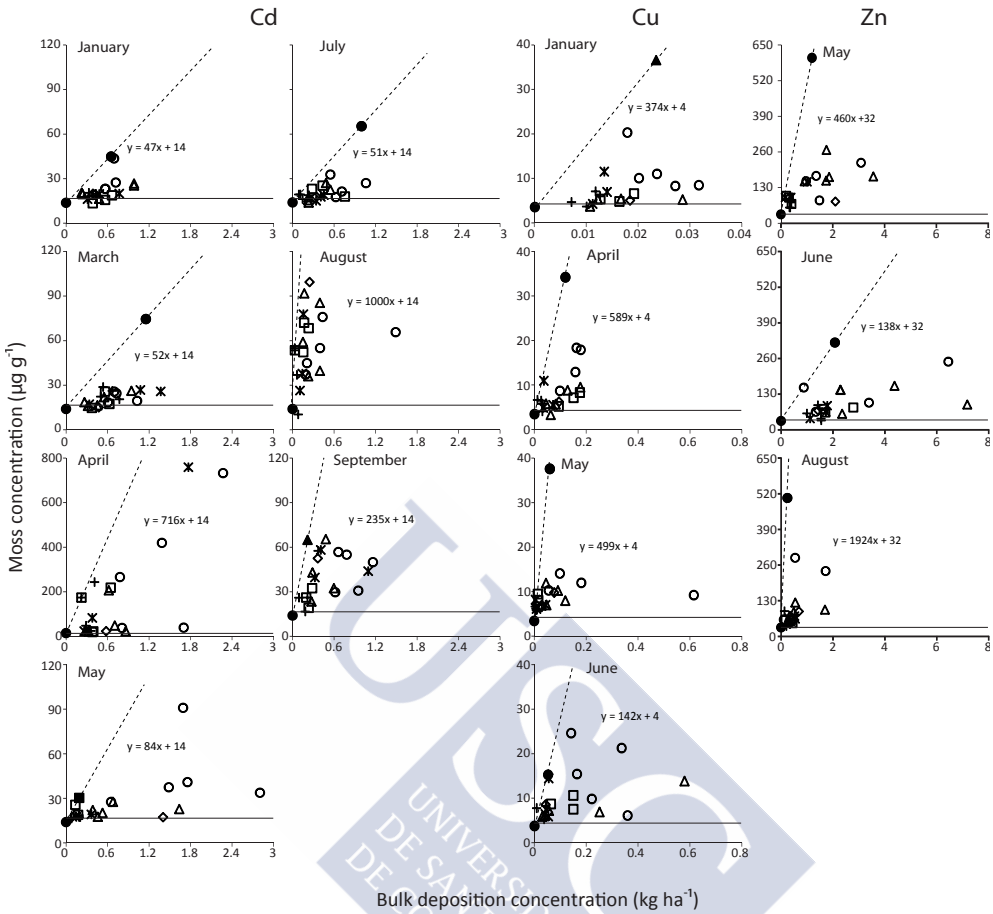


Fig. 3. Graphical representation of the concentration of Cu in *Sphagnum denticulatum* in relation to bulk deposition grouped by sampling site (SS). Limits of quantification of the technique (LOQ_T) are represented by a solid black line, whereas the straight line of maximum slope is shown as a black dashed line. The equation for the line is included in the graph. Squares= winter, triangles=spring, circles=summer, asterisk=autumn.

even weekly monitoring periods (Aas et al., 2009).

Secondly, the standard uncertainty is one of the most important metrological concepts in the validation of analytical methods and implies a measure of quality in the result. Moreover, the 4th Daughter Directive states that an uncertainty of less than 70 % should be the data quality objective for total deposition of Ni, Cd and As. In the present study, we applied the uncertainty term in the field of biomonitoring because the overall aim is to produce a standardized protocol for the moss bag technique so that it can be routinely applied by public authorities as a reliable tool for estimating air quality. Although the standard uncertainty is acceptable in both types of samplers, according to the EU requirements, the results indicate that for all elements measured, the uncertainty was lower for the moss bags than for the bulk deposition. The many sources of uncertainty in the deposition measurements depend on the measurement methods used in both the laboratory and in the field

(Aas et al., 2009).

In a previous study by reviewing the correlations founded in the literature of the concentrations of elements in moss samples and in bulk deposition, Aboal et al. (2010) found high correlations, especially for Pb, Cd, Hg and, to a lesser extent, Cu and Zn in native moss; in contrast, we found a higher proportion of significant correlations in the moss bags and bulk deposition for Cu, Pb and Zn and fewer significant correlations for Cd (Table 2). More specifically, in the present study, we detected a low number of significant correlations: 21% for Cd, 18% for Cu and 9% for Zn. However, grouping the data on the basis of the exposure time and comparing the bulk deposition and moss concentration data revealed a greater number of significant correlations than when the data were grouped by SS. The element for which the greatest number of correlations were observed was Cd, which is consistent with the findings of other studies carried out with native moss (Berg and Steinnes 1997; Schintu et al., 2005).

The possible causes of variability must be taken into account in considering the correlations obtained. Thus, if the data are grouped by SS, the type of emission (e.g. gaseous, particulate, particle size, etc.) will be homogeneous and therefore the variability will depend on the changes in the atmospheric conditions and/or small changes in the emissions. On the other hand, when the data are grouped by exposure period, the variability regarding the atmospheric conditions decreases, but the variability associated with the type, amount and general characteristics of the emission increases. Therefore, if the moss preferentially accumulates one type of emission, the variability in the data will increase.

The results grouped by SS only showed correlations for Cu at two sites: SS3 (coal-fired power plant) and SS11 (steelworks). The range of concentrations obtained in other similar studies is shown in Table 2, as this may influence the presence or absence of correlation. The maximum and minimum values shown in Table 2 are those found for each data set used in the correlation analysis. In the present study, 21 correlations were determined (i.e. one for each SS) and the total number of data points is 12 (corresponding to the number of months that the experiment lasted). Although comparison of the different studies is hampered by the use of very different methods regarding the preparation and exposure of the moss bags (e.g. ratio of moss weight to surface area of the bag, species used, etc.), the range of concentrations was higher than that found in previous studies (Table 2). The differences in uptake of contaminants may be related to the different weights of moss per unit of surface area used in the different studies (between 12.5 and 15 mg cm^{-2} compared with the 2.5 mg cm^{-2} used in the present study). We recently demonstrated that uptake is higher at a low ratio of weight of moss per surface area of the mesh bag (Ares et al., 2014). Therefore, for the same emission characteristics, the lack of correlation for some elements may be due to differences in weather conditions, including precipitation, which has an important influence on uptake. As significant correlations were found for Cu in 2 SS, it is possible that the effect of changes in the environmental conditions have less effect on this metal.

On the other hand, the greater number of significant correlations in the data grouped by exposure period may be related to more homogeneous environmental conditions. Thus, although there were some differences between the SS, they were all in the same region and subjected to the same precipitation and temperature regimes. Two periods can be identified on the basis

Table 2. Minimum and maximum concentrations recorded in moss and bulk deposition in the present and previous studies. Significant correlations ($p<0.05$) for the moss concentrations are shown in bold type. All the correlations were analysed/determined by Spearman's rank test, except for Pilegaard (1979), in which linear regression was applied. For Duggan and Burton (1983), we calculated the correlation with the data provided in the paper. The concentrations of elements in moss are expressed in μg g^{-1} (except * ng g^{-1}) and in bulk deposition, in kg ha^{-1} (except * g ha^{-1}).

				Moss										BD									
Study		**n**	**t**	**Cd***		**Cu**		**Hg***		**Pb**		**Zn**		**Cd***		**Cu**		**Hg***		**Pb**		**Zn**	
				Min	**Max**	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**
Aničić et al (2009)		15	4	0.19	0.36	**10**	**19**	-	-	**7**	**38**	**44**	**105**	0.0126		0.0180		-	-	0.0177		0.0183	
Duggan and Burton (1983)		9	4	**0.4**	**1.6**	**8**	**40**	-	-	**24**	**200**	**12**	**480**	0.0005	0.001	0.0015	0.02	-	-	0.02	0.15	0.06	0.3
Pilegaard (1979)		12	28	**0.1**	**6.6**	**1.15**	**63.75**	-	-	**6**	**493**	**22**	**1579**	0.001	0.066	0.015	0.637	-	-	0.06	4.933	0.228	15.79
Present study	SS1	12	4	17.47	577.34	5.08	18.01	41.99	85.49	77.75	636.22	51.5	664.2	0.246	1.405	0.018	0.095	0.004	0.297	0.005	2.996	0.549	2.996
	SS2	12	4	13.55	99.40	4.11	11.34	41.55	78.55	94.42	511.03	28.0	177.2	0.150	0.515	0.009	0.057	0.015	0.095	0.006	2.000	0.331	4.374
	SS3	12	4	13.80	64.89	5.17	14.85	44.36	80.93	70.90	728.23	74.4	832.4	0.206	0.864	0.013	0.083	0.001	0.426	0.006	3.847	0.258	3.847
	SS4	12	4	14.38	65.37	**3.43**	**10.45**	41.16	71.56	72.41	562.40	40.2	154.8	0.105	0.393	0.010	0.064	0.003	3.138	0.006	2.144	0.549	2.298
	SS5	12	4	13.63	77.73	4.22	11.23	35.22	68.66	62.36	661.42	27.1	150.3	0.393	1.638	0.026	0.252	0.004	0.310	0.024	2.909	1.408	7.176
	SS6	12	4	16.80	91.74	3.31	14.28	39.05	69.48	52.50	600.97	44.2	237.8	0.226	1.653	0.023	0.579	0.004	0.187	0.025	1.031	0.353	2.840
	SS7	12	4	17.11	721.50	5.18	36.33	42.36	75.43	113.35	428.02	81.1	215.0	0.209	2.804	0.016	0.615	0.001	0.735	0.017	1.026	0.103	1.541
	SS8	12	4	25.65	359.96	10.99	31.98	43.98	493.53	95.05	480.69	71.7	286.1	0.174	1.486	0.024	0.227	0.004	0.194	0.027	1.663	0.521	3.065
	SS9	12	4	37.12	732.25	20.27	59.20	55.86	1736.61	74.78	1423.27	98.0	603.0	0.007	2.271	0.018	0.201	0.008	0.720	0.015	2.805	0.246	2.805
	SS10	12	4	24.13	432.03	10.03	27.20	44.40	321.43	69.16	265.16	93.1	248.3	0.342	2.529	0.020	0.337	0.004	5.178	0.027	5.316	0.542	6.445
	SS11	12	4	18.44	318.90	**7.94**	**21.61**	46.81	267.25	55.12	327.60	61.4	153.4	0.127	1.514	0.027	0.347	0.003	0.178	0.027	4.725	0.441	4.725
	SS12	12	4	25.59	66.05	6.22	36.15	44.09	157.67	84.76	1159.53	85.0	269.9	0.167	0.987	0.013	0.249	0.002	0.097	0.008	1.852	0.209	2.016
	SS13	12	4	13.36	72.05	3.82	9.53	39.50	89.68	78.70	871.87	34.0	75.1	0.051	0.861	0.014	0.176	0.001	0.069	0.014	1.371	0.177	2.788
	SS14	12	4	15.41	465.52	5.50	10.84	40.62	76.93	110.16	820.17	42.6	262.9	0.044	1.049	0.016	0.239	0.004	0.136	0.012	0.953	0.214	3.683
	SS15	12	4	13.69	55.19	4.80	10.37	37.12	76.74	88.20	690.73	44.9	99.6	0.037	0.411	0.011	0.149	0.009	0.157	0.009	6.798	0.546	6.798
	SS16	12	4	13.66	59.09	3.76	15.25	38.23	72.78	71.91	1006.68	37.6	127.2	0.107	1.369	0.009	0.178	0.002	0.116	0.007	2.085	0.295	2.033
	SS17	12	4	15.17	72.74	4.64	8.43	44.75	67.27	62.38	342.85	42.7	233.1	0.011	2.528	0.005	0.125	0.006	0.248	0.004	1.893	0.174	2.085
	SS18	12	4	10.22	683.18	4.19	8.24	42.04	98.85	79.90	528.72	33.4	73.0	0.063	1.841	0.008	0.071	0.004	0.139	0.003	1.344	0.344	1.419
	SS19	12	4	12.61	388.07	3.64	8.83	37.38	80.10	89.45	684.28	28.7	91.5	0.028	0.668	0.004	0.044	0.000	2.068	0.001	1.174	0.165	1.344
	SS20	12	4	14.85	82.25	3.89	10.21	37.23	80.29	77.49	424.88	40.6	93.7	0.031	0.772	0.007	0.077	0.001	0.084	0.003	2.033	0.133	1.548
	SS21	12	4	16.42	758.56	5.50	52.94	41.18	88.14	63.98	330.81	46.3	104.1	0.080	0.838	0.010	0.112	0.003	0.129	0.011	1.411	0.206	7.024

of the amount of precipitation across all SS: i) a wet period between October and March and ii) a dry period between April and September (Table 1). The significant correlations were mainly recorded in the driest months of the year (Fig. 3). The occurrence of rain may influence the uptake of contaminants by moss in opposite directions: if moderate it may favour removal of contaminants from the atmosphere and their capture in the moss bags. However, heavy rainfall (particularly if slightly acidic) may lead to leaching of metals and removal of particles (Tavares and Vasconcelos, 1996). Čeburnis and Valiulis (1999) calculated that the retention efficiency of *Hylocomium splendens* and *Pleurozium schreberi* for the studied metals was highly variable (range 0 to 92%, with Pb and Cu retained to the highest degree and Cd and Zn to the lowest degree), and that the highest retention efficiencies occurred in June, July and August, when the rainfall was lowest. The results found on comparing the residual concentrations in the significant correlations and the rainfall levels in the present study were positively and significantly correlated at several sites. On the other hand, the differences between characteristics related to the type of emission (i.e. gas or particulate) had less influence, at least for Cd.

Another reason for the lack of correlation may be that in the present study (as in previous studies with both native moss and moss bags), only the elements present in the soluble fraction of the bulk precipitation have been considered. The contribution of the particulate fraction of the bulk deposition should also be considered to determine whether it is correlated with the concentration in moss. Previous studies carried out with moss bags have shown a relation between airborne particles in dry deposition and moss content (Vuković et al., 2014), as moss is efficient at retaining particles (Tretiach et al., 2011; Spagnuolo et al., 2013) and may even retain up to 98% of the PM_{10} particles from the air. We may therefore expect, a priori, a higher correlation between the concentrations in moss and this fraction of the bulk deposition. In a study of atmospheric inputs of soluble and particulate trace elements in the Mediterranean basin, Theodosi et al., (2010) used wet and bulk deposition samplers and determined the proportion of elements associated with each of the fractions. These authors pointed out that while V, Cr, Fe and Pb were mainly associated with the particulate form (64–98%), the soluble fraction of Mn, Zn, Cu and Cd represented 60–70% of the total input. However, the presence of the soluble or insoluble fraction of a particular metal will depend on numerous factors that interact together, including pH, amount of rainfall, time of year and dust load (Castillo et al., 2013; Tanner and Wong, 1999, Theodosi et al., 2010).

The maximum slopes represented in Figs. 2 and 3 indicate the maximum capacity of the moss to accumulate trace elements in relation to minimum amounts of these elements in the bulk deposition. With one exception (Cd in August, Fig. 3.), the values are located below this line and are highly dispersed. This indicates that other factors affect the mechanisms of element accumulation in mosses. The uptake and retention of elements may also be influenced by the morphological features of moss and by atmospheric conditions (i.e. wind frequency and speed, precipitation frequency intensity and duration) (Aničić et al., 2009). Although the contamination is integrated in the bulk deposition, mosses do not retain or accumulate elements indefinitely and the contents of elements will be strongly influenced by recent environmental conditions, thus explaining some of the losses.

5. Conclusions

The experimental design of the present study proved suitable for achieving the objectives of the study in terms of contaminant concentrations and standard uncertainty values. As regards the latter, the level of uncertainty was lower than in the bulk deposition in all cases.

Within the theoretical framework of the moss bag technique, the present study provides further information about the type of relationship between the concentrations of contaminants in moss and those in the soluble fraction of the bulk deposition.

Most of the significant correlations involved Cd and to a lesser extent Cu and Zn. The environmental conditions and particularly the abundance of precipitation are the main causes of the absence of correlations in some cases, presumably because of the loss of elements by leaching. Indeed, most of the significant correlations were observed in the driest months. The type and amount of emission were important in relation to the presence of correlations between the elements under study. The total lack of correlations for Pb and Hg indicates the need for further studies to determine the relationship between the concentration of trace elements in moss bags and the particulate fraction in the bulk deposition. However, despite the limits of the method must always be borne in mind, the moss bag technique is a very useful and economical environmental tool

Acknowledgements

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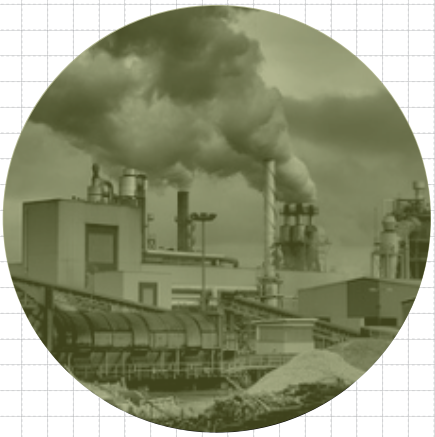
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Chapter IV



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Study of the air quality in industrial areas of Santa Cruz de Tenerife (Spain) by active biomonitoring with *Pseudoscleropodium purum*

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Abstract

A biomonitoring technique with terrestrial moss transplants (50 sampling sites in a regular grid) was used in an area of the city of Santa Cruz de Tenerife, close to an oil refinery and to an area of dense road traffic for a period of 2 months. The concentration of metals and metalloids (As, Cd, Hg, Ni, Pb and V) and 16 polycyclic aromatic hydrocarbons (PAHs) were determined. The density distribution was represented, the enrichment factors calculated and multifactorial analysis applied. In addition, contamination maps were elaborated on the basis of the bioconcentration obtained, and after confirming the existence of spatial structure, the response surfaces were represented. The results showed very high levels of contamination by Ni and V in the study area, with similar dispersal patterns observed for both. The concentrations of Cd, Hg, Pb and PAHs were lower. Active biomonitoring with terrestrial mosses was found to be a suitable technique for implementing inexpensive environmental monitoring programmes in urban and industrialized areas.

Keywords: Urban environment, Moss bags, Heavy metals, Polycyclic aromatic hydrocarbons.

Introduction

Urban environments are subject to intense contamination of anthropogenic origin, which may involve static sources, such as factories and domestic heating, or mobile sources, such as road traffic (Aničić et al., 2009). Certain contaminants, such as polycylic aromatic hydrocarbons (PAHs) and heavy metals, are therefore widely distributed in such environments. These contaminants may be toxic and have serious effects on human health (Hinwood and Di Marco, 2002; Valavanidis et al., 2005) and the measurement of the amounts and dispersal of these contaminants has been the object of many studies (eg Nriagy and Pacyna, 1988).

Physical and chemical methods are commonly used in studies of atmospheric contamination to measure the concentrations of contaminants, and often involve sophisticated, expensive techniques. This has led to increased interest in biomonitoring

with terrestrial mosses in recent years. The latter technique enables the design of sampling grids best adapted to each environment and study, and the simultaneous measurement of several different types of contaminants (both micro and macrocontaminants).

In urban and industrial environments where native mosses may be scarce or even absent, the moss transplant technique ("moss bag technique") has been developed in order to measure the bioconcentration of contaminants (Goodman and Roberts, 1971; Tyler, 1990). There are several advantages associated with this technique, including the uniformity of the surface of uptake, and the fact that the initial concentrations of the contaminants in the moss are known and that the time of exposure can be predetermined (Steinnes, 1989). These advantages indicate the suitability of the moss bag technique in intensive studies in urban and industrial environments, as the concentrations of contaminants in the affected area can be estimated and the patterns of dispersal described. However, the technique has not been completely standardized, in terms of the amount of moss exposed in the bags, and the shape of the bags and the material used to make them; there is also a lack of information as regards the correlation between the deposition of contaminants and their uptake by moss. Another important aspect, not still standardized, is the treatment of the blanks or zero time controls. The results are generally expressed in terms of such concentrations, which are subject to spatial and temporal variability. Couto et al., (2004) in a study with irrigated transplants recommended that all active biomonitoring studies should include a series of blanks that enable calculation of the limit of detection (LOD) and the limit of quantification (LOQ) of the transplant technique, for correct interpretation of the results. We followed these recommendations in the present study, and in addition to calculating the LOQ of the technique, we used additional moss bags as controls to check for possible contamination during laboratory handling and movement of the transplants, in order to increase the sensitivity of the method this novel aspect allows a better quality of results to be obtained.

Studies with active biomonitoring of heavy metals, have been carried out successfully in cities such as Belgrade (Aničić et al., 2009), Budapest (Naszradi et al., 2007), Naples (Giordano et al., 2005), Oporto (Vasconcelos and Tavares, 1998) and Sofia (Culicov and Yurukova, 2006), amongst others. However, few studies have implemented such a high density of sampling site (SS) as in the present study, which has enabled a complete assessment of the study area using for the first time geostatistical tools.

On the other hand very few studies have been carried out with regard to active biomonitoring of PAHs with moss transplants, only a few in industrial areas (Wegener et al., 1992) and in areas with heavy traffic (Viskari et al., 1997 and Zechmeister et al., 2006), but none in urban areas. Simultaneous determination of PAHs and heavy metals in these environments is another novel aspect of the present study. These compounds are widely distributed in such environments and are harmful to human health.

The aim of this study, is to employ the technique of active biomonitoring with terrestrial mosses to study dispersion patterns of pollutants in urban environments. Therefore the concentrations of metals and metalloids (As, Cd, Hg, Ni, Pb, and V) and polycyclic aromatic hydrocarbons (PAHs) were, firstly in the available literature simultaneously determined in samples of the terrestrial moss *Pseudoscleropodium*

purum placed in an area of the city of Santa Cruz de Tenerife close to an industrial zone and to an area of dense road traffic. And finally, the use of geostatistical tools, for the first time, to obtain important information such as the possible existence of spatial structure of the different pollutants as well as the response surface.

2. Material and methods

2.1. Study area and experimental design

The study area is in the city of Santa Cruz de Tenerife (Canary Islands, Spain), in a zone close to the city's industrial area and to an area with very dense road traffic. The sampling design was a regular grid of 400x400 m, with 50 sampling sites (SS) (Fig. 1). At each of the sites in the grid, the moss transplants were attached to the closest lamp post free of plant cover. Most sites in the grid that coincided with areas covered by the sea were abandoned, although some were eventually relocated to nearby coastal areas.

2.2. Preparation of the transplants

Selection of the moss species is another key aspect that must be standardized in the "moss bag" technique. For the present study we chose to use the terrestrial moss *Pseudoscleropodium purum* (Hewd.) M. Fleisch. This species is easy to identify and to handle in the laboratory, and also has a large specific surface area and a wide spatial and temporal distribution. It has been widely and successfully used in active biomonitoring studies with moss bags (e.g. Castello, 2007; Couto et al., 2004). It also occurs naturally on the Island of Tenerife.

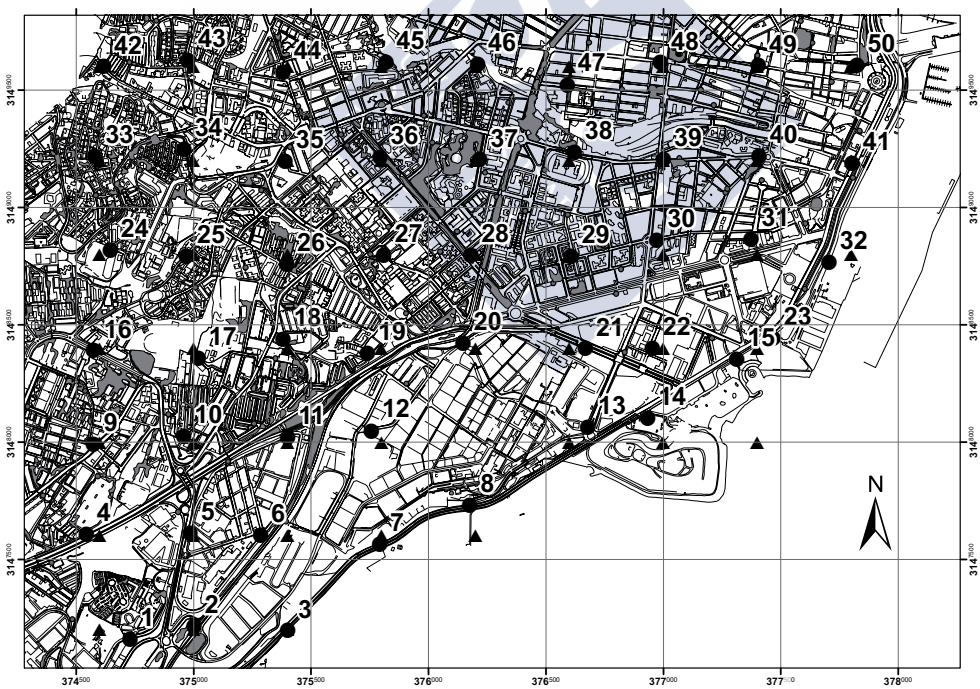


Fig. 1. Map of the active biomonitoring network implemented with the terrestrial moss *Pseudoscleropodium purum* in the city of Santa Cruz de Tenerife. The triangles represent the theoretical grid, and the circles represent the sites where the transplants were finally placed.

Samples were collected from a uncontaminated area (Aboal et al., 2004. In the laboratory, apical sections (3-4 cm) were separated from the shoots, placed in a plastic sieve (0.7 cm mesh size) and washed with bidistilled water (for 30 min with shaking) to eliminate plant remains, epiphytes, adhering soils particles, etc. The washing enhanced activation of the moss as it eliminated elements bound to cation exchange sites on the cell wall and membrane. After the washing stage, excess moisture was removed with filter paper and 10 g of the material (fresh weight) was weighed and then was introduced into the bags for transplanting.

Bags (10 x 20 cm) were prepared with polythene netting (1mm mesh), previously washed in HNO_3 to eliminate possible trace contaminants. The moss was placed evenly inside the bags and a rigid polythene bar was inserted to prevent the bags and their contents being deformed by the wind. Finally, each bag was hung on a PVC tube attached perpendicular to the lamp post, at a height of 3-4 m.

A total of 57 moss bags were prepared; 50 of these were placed in situ between 18th March and 20th May 2008, 3 were controls that were treated in the same way as the transplants but were not exposed (i.e. the same laboratory handling and journeys) and 4 were blanks which were vacuum packed prior to the period of exposure

Table 1. Limit of quantification of the transplant technique (LOQ_T) and descriptive statistics regarding metalloids, metals and PAHs following the determination of the concentrations of these in transplants of *Pseudoscleropodium purum* after two months of exposure at the 50 sampling sites (SS).

	LOQ_T	Blanks	Mean	SD.	Max.	Min.	Skew	Kurtosis
As	465	149	390	168	780	77.8	0.0818	-0.572
Cd	266	101	420	226	1545	161	3.19	13.4
Hg	60.2	31.3	84.8	18.8	121	45.4	0.104	0.570
Ni*	4.80	0.790	7.88	4.55	23.8	2.66	1.76	3.16
Pb*	2.79	1.80	6.16	3.43	17.8	2.56	1.76	2.80
V*	1.47	0.101	8.68	4.56	20.2	2.00	0.566	-0.410
Naf	860	465	259	136	591	18.5	0.224	-0.108
Ace	25.4	14.8	15.0	14.1	106	5.82	5.76	36.8
Fl	39.8	17.8	15.1	17.4	132	6.90	6.50	44.4
Fen	186	40.8	60.6	21.0	109	28.1	0.788	-0.0812
Ant	17.6	4.95	6.06	2.28	10.7	1.55	0.232	-0.626
Flu	20.4	24.0	57.6	30.6	148	19.8	1.46	1.68
Pir	75.7	37.4	208	333	1954	28	4.40	20.2
BaA	5.06	6.77	5.30	4.34	20.9	1.22	2.14	4.54
Cr	18.2	7.50	14.5	7.88	40.9	4.14	1.48	2.40
BbF+BjF	113	14.8	25.4	13.6	71.7	3.82	1.02	1.52
BkF	296	10.4	15.2	28.3	184	1.16	4.85	26.9
BeP	66.0	16.6	33.0	22.0	102	3.48	1.13	1.16
Bap	82.0	4.04	14.5	21.2	100	0.534	2.48	6.10
BghiPe	188	1.74	4.95	7.17	37.9	0.310	3.26	11.3
DahA	254	3.70	10.0	12.1	54.4	0.490	2.28	5.15
InP	234	12.9	48.5	45.5	273	1.18	2.63	10.9

The concentrations are expressed in ng g^{-1}, except for those marked with *, expressed in µg g^{-1}. SD: standard deviation. Naph: Naphthalene; Ace: Acenaphthene; Fl: Fluorene; Phen: Phenanthridine; Ant: Anthracene; Flu: Fluoranthene; Py: Pyrene; BaA: Benzo(a)anthracene Cr: Crysene; BbF+BjF: Benzo(b)fluoranthene+Benzo(j)fluoranthene; BkF: Benzo(k)fluoranthene; BeP: Benzo(e)pyrene; BaP: Benzo(a)pyrene; BghiPe: Benzo(ghi)perylene; DahA: Dibenzo(a,h) anthracene; InP: Indopyrene.

to avoid any possible contamination. This enabled us to control for any possible contamination of the moss during the laboratory handling and during transport.
In the laboratory, the moss in each of the bags was homogenized in an ultracentrifuge mill (Restch ZM 200, heavy metal-free) without prior washing to prevent the loss of particulate fraction. Then, the samples were dried at 80º C (for determination of Hg, an aliquot of the homogenate was dried at 40º C) until their chemical analysis.

2.3. Chemical analysis

2.3.1. Determination of metals and metalloids

The metals chosen for analysis were As, Cd, Hg, Ni and Pb as they are included in Council Directive 96/62/EC and 'daughter' directives relating to ambient air quality, including European Parliament Directive 2004/107/EC relating to As, Cd, Hg and Ni, and Directive 1999/30/EC relating to Pb (both of which have been transposed to Spanish legislation). Finally, V was chosen as it is closely linked to the oil industry.
The concentrations of As, Cd, Ni, Pb and V in solid suspensions were measured by graphite furnace atomic absorption spectrophotometry (Perkin Elmer AAnalyst 600). An element analyzer (Milestone DMA 80) was used for determination of Hg.
To control the quality of the analysis, analytical replicates were processed, 1 each 10 samples, and the corresponding standard deviations were calculated, in accordance with the method of Čeburnis and Steinnes (2000). Certified reference material (M2 and M3) (Steinnes et al., 1997) corresponding to the moss *Pleurozium schreberi*, was also analysed, 1 every 10 samples. Contamination during processing was also controlled for by the use of analytical blanks, 1 every 10 samples analysed.

2.3.2. Determination of PAHs

The PAHs were determined at the Institute for the Environment at the University of A Coruña, following the protocol described by Ares et al. (2009). Briefly, a solution of standard deuterised PAHs (napthalene d-8, acenaphthalene d-10, phenanthrene d-10 and crysene d-12) dissolved in acetone were added to each sample, and a mixture of hexane/acetone was added. Sample extraction was carried out in a microwave oven (ETHOS SEL, Milestone). The samples were purified on glass columns with silanized glass wool, Florisil® and gel. The extracts were concentrated to a drop on a rotavapor to remove the dissolvent. Finally, the PAHs were determined by gas chromatography-mass spectrophotometry in MS/MS mode.
The following PAHs were determined: naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, crysene, benzo(b)fluoranthene + benzo(j)fluoranthene, benzo(k)fluoranthene, benzo(e)pyrene, benzo(a)pyrene, benzo(ghi)perylene, dibenzo(a,h)anthracene, and indopyrene.

2.4. Data analysis

The limit of quantification of the technique was calculated for the first time in accordance with the method described by Couto et al. (2004). The enrichment factors (EFs) were calculated as the ratio between the concentrations at the end of the exposure period and the initial concentrations. The mean value of the blanks concentrations was considered as the initial concentration (Couto et al. 2004).
To calculate the density distributions, was used the KernSmooth statistical package

(Ripley, 2002) in the free software R (R Development Core Team, 2002). Kernel smoothing was applied by selecting a Gaussian kernel with the width for each data set optimized by the technique of direct introduction with two levels of functional estimation (Wand and Jones, 1995). The combined treatment of the contamination data was carried out by principal components analysis (PCA), with a Varimax rotation, following the procedures in SPSS version 15.0.

The spatial structure was first studied by elaboration of the robust semivariogram for regular lags (Cressie and Hawkins, 1980), considering the null hypothesis of absence of spatial structure in the data, and a randomization test of the positions and concentrations determined was carried out (with 10000 repetitions) (for details, see Aboal et al., 2006). When spatial structure was observed (rejection of the null hypothesis), the polynomial functions that described the response surface that best fit the data obtained from the sampling grid were calculated (Real et al., 2003). Those models with all terms significant at $P > 0.05$ and which also absorbed as much variation in the data as possible (fitted R^2), were selected. The Statgraphics 5.1 program was used for the regression and polynomial analyses.

The maps showing the locations of the transplanted samples, and of the bioconcentration corresponding to each contaminant, were elaborated with the ArcGIS 9.2 program.

3. Results

3.1. Analytical quality

The recoveries of heavy metals and metalloids from the reference materials were satisfactory and close to 100%, except for As in M3, for which recovery was 198%. The overall error associated with the analytical process varied between 14% for As and 4% for Hg. As regards inorganic contaminants, the limit of quantification of the analytical technique ranged between 0.31 and 12.1 ng g^{-1}, for Cd and V respectively. The limit of quantification of the transplant technique varied between 59 ng g^{-1} and 4.78 μg g^{-1} for Hg and Ni respectively (Table 1).

The percentages of recovery for the PAHs were higher than 90% for all compounds except naphthalene (60%). Relative standard deviations between duplicates were below 10%. The analytical limits of quantification varied between 9.1E-05 for benz(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene + benzo(j)fluoranthene, benzo(e)pyrene, benzo(k)fluoranthene, benzo(ghi)perylene, dibenz(ah) anthracene, crysene and indopyrene and 0.014 ng g^{-1} for naphthalene. The limits of quantification of the transplant technique for these compounds ranged between 5 ng g^{-1} for benz(a)anthracene and 862 ng g^{-1} for naphthalene. The limits of quantification of the technique for each of the contaminants are shown in Table 1.

3.2. Concentrations of the contaminants analysed

On collecting the samples it was noted that they were blackened with soot, especially those collected from close to the soil refinery, and therefore many contaminants may have adhered to the soot particles.

The descriptive statistics corresponding to the determination of the concentrations of heavy metals, metalloids and PAHs are shown in Table 1.

All of the concentrations of V measured were higher than the limit of quantification of the technique. In the case of Cd, Hg, Pb and Ni, few of the concentrations were below the limit of quantification (Table 1). For As, most of the concentrations were below the limit of quantification of

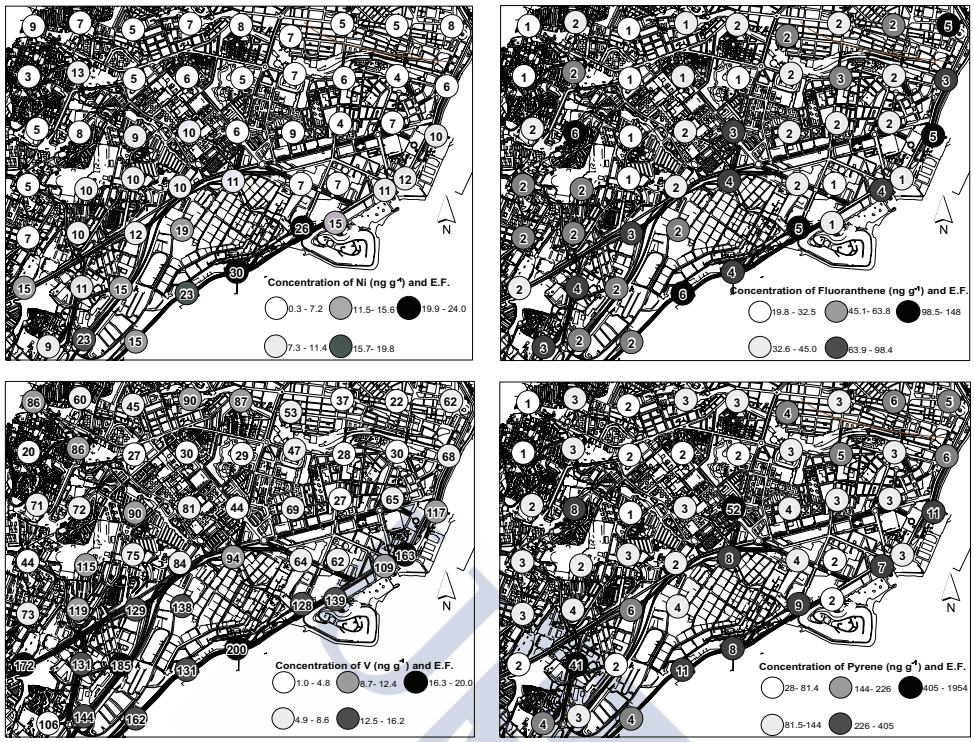


Fig.2 Enrichment factors (EF) and tissue concentrations in the terrestrial moss *Pseudoscleropodium purum* after two months of exposure in the city of Santa Cruz de Tenerife. Data for the metals Ni and V, and the PAHS fluoranthene and pyrene are shown.

the technique and therefore no quantitative data were obtained for this metal as the concentrations could not be differentiated from the initial concentrations (at time zero).

The highest variances and ranges corresponded to Ni, Pb, and V (Table 1). The most skewed distribution corresponded to Cd, followed by Ni, Pb and V; all of the skew values were positive, except that for Hg.

Many of the concentrations of most of the PAHs, except fluoranthene and pyrene, were below the limit of quantification of the technique (Table 1).

3.3. Enrichment factors (EFs)

By way of example, the concentrations of Ni, V, fluorene and pyrene and the corresponding enrichment factors are shown in Fig. 2, as these were the highest obtained of all of the contaminants considered. For V, the EFs reached values as high as 200, and for several samples the EFs were higher than 100. The EFs obtained for the PAHs were generally much lower than those obtained for metals.

3.4. Density distributions

The kernel smoothed density distributions obtained for heavy metals and some of the PAHs are shown in Fig. 3.

As regards the heavy metals and metalloids, the distributions of Pb, Ni and Cd show atypical values, with large deviations from the normal distribution. For example the data corresponding to Cd include an extreme value 4 times higher than the distribution (SS 3). In the case of Pb, the extreme value was 4 times higher than the

modal value (SS 4), and for Ni, 5 times higher (SS 8). Some of the atypical values in the density distributions are the same for the different elements determined.

The highest concentrations of Cd obtained corresponded to samples 3 and 14; those of V to samples 8, 2, 6 and 14; Ni to sites 8, 13, 2 and 7 and to a lesser extent, those of Pb to samples 4, 32 and 23. Thus the highest concentrations of these elements corresponded mostly to the first 10-15 samples (Table 1). The proximity of the samples within the sampling grid may indicate the existence of spatial structure.

The most skewed distributions were those of Hg and As. This may indicate a lack of contamination by these elements, however, as already mentioned the As data cannot be used quantitatively.

The density distributions corresponding to some of the PAHs determined are also shown (Fig. 3). Of those samples with extreme values, sample 23 is particularly noteworthy, as most of the compounds shown in Fig. 3 were detected in this sample. In terms of extreme values, this sample is followed by transplants 7, 8 and 32. For some of the compounds the extreme values occur in the same order (i.e. acenaphthalene and fluorene; phenanthene and fluoranthene) in different samples, which may explain why these compounds were grouped in the same factor in the principal components analysis. There was little correspondence between those samples in which extreme values of metals and metalloids (except for Pb) were observed.

3.5. Multifactorial analysis

The first 4 factors obtained in the PCA accounted for 61% of the total variance in the data, with 21% corresponding to the first factor, 18% to the second, 13% to the third and 9% to the fourth. The component matrix obtained after Varimax rotation is shown in Table 2.

3.6. Spatial structure

The robust semiovariograms for Ni, V and benzo(b)fluoranthene +benzo(j)fluoranthene are shown in Fig. 4, as examples of those obtained for metals, metalloids and PAHs.

The first lags in all of the semivariograms for heavy metals and metalloids were significant (i.e. below the 2.5% quantile).

In the case of Ni, Pb and V the first 4 lags were significant, so that the null hypothesis of the non existence of spatial structure can be rejected for these elements. Therefore the semivariograms would be approximately linear, with the value of the variance increasing with the lag distance. This would indicate a response surface similar to an inclined plane. However, for As and Hg, except for the first lag, the robust semivariance is within the region of acceptance of the null hypothesis, and therefore these two elements possibly do not show any spatial structure.

Except for anthracene, crysene and benzo(b) fluoranthene + benzo(j)fluoranthene (Fig 4), there was no spatial structure in the concentrations of the other PAHs.

3.7. Response surfaces

To estimate the surface response for the different contaminants, those polynomials with maximum fitted coefficient of determination (R^2aj), were selected from the existing possibilities after it was found that all terms in the polynomial were significant and the residuals generated did not display any remnant spatial structure. If there is spatial structure, this would indicate poorer quality of the response surfaces found. There was no spatial structure in the residuals of any of the contaminants (Fig. 4).

As regards heavy metals and metalloids, polynomials were obtained for Ni, with all terms significant at $p \leq 0.01$, and for the

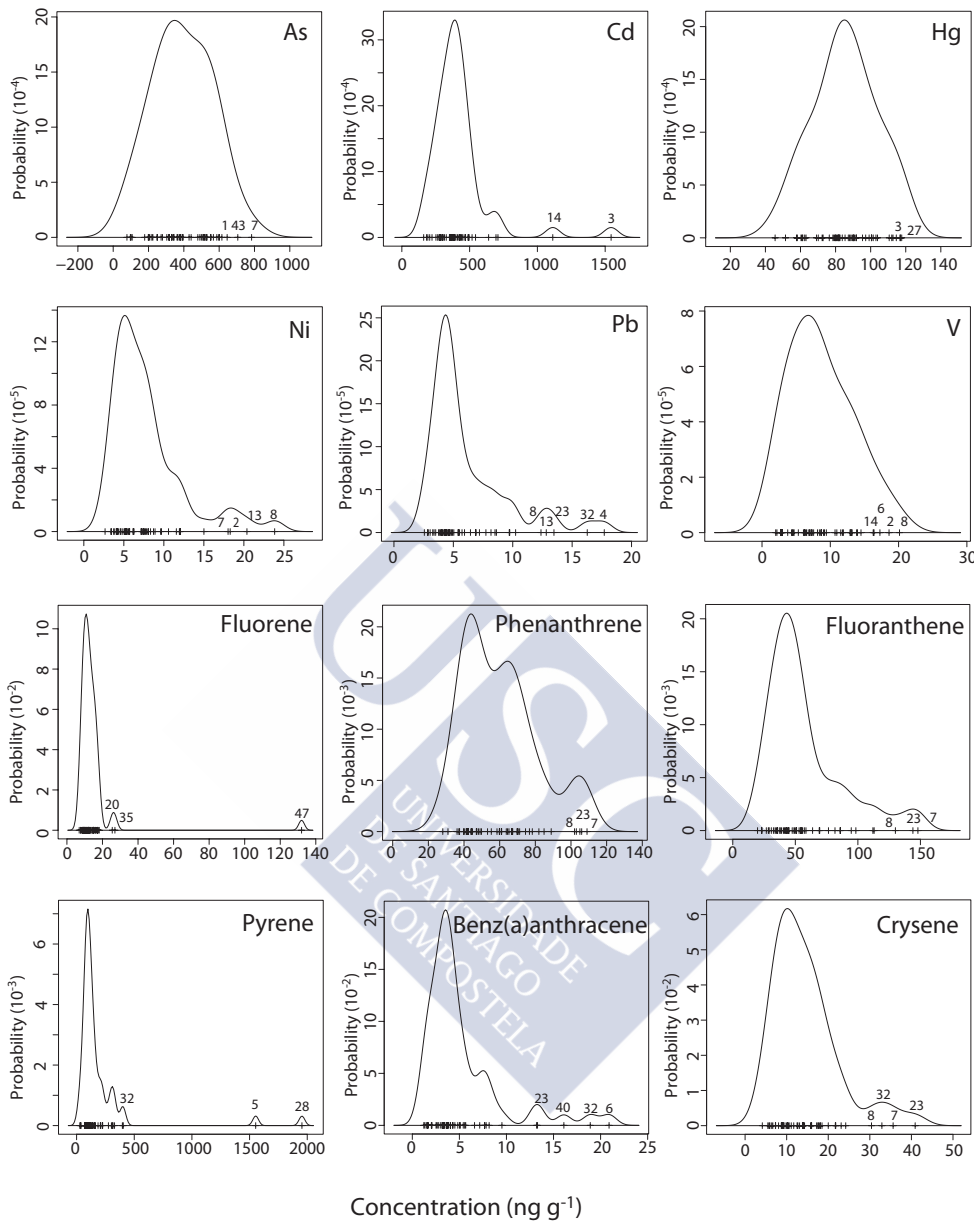


Fig. 3. Density distributions obtained after application of kernel smoothing to the concentrations of metals and PAHs (fluorene, phenanthene, fluoranthene, pyrene, benzo(a)anthracene and crysene), in the terrestrial moss *Pseudoscleropodium purum* after two months of exposure. The samples corresponding to extreme values are indicated on the graph..

other elements, with all terms significant at p≤0.05. The surface responses for Ni and V, the metals with the polynomials with the highest coefficients of determination (69 and 71%, respectively) are shown in Fig. 4.

The coefficients of determination were much lower for the PAHs than for the other compounds, and the compound for which the explained variance was highest was benzo(b)fluoranthene+ benzo(j) fluoranthene (approximately 30%) (Fig. 4).

Table 2. Factor matrix obtained by principal components analysis (PCA) of the 50 moss transplants and the metals, metalloids, and PAHs determined. Absolute values lower than 0.4 are not shown.

Element/compound	Factor 1	Factor 2	Factor 3	Factor 4
As				
Cd		0.628		
Hg		0.746		
Ni		0.822		
Pb		0.725		
V		0.932		
Naf				
Ace				0.986
Fl				0.977
Fen	0.527		0.634	
Ant		0.432	0.716	
Flu	0.469		0.659	
Pir			0.759	
BaA	0.613			
Cr	0.612	0.435	0.539	
BbF+BjF				
BkF	0.917			
BeP	0.828			
Bap				

Naph: Naphthalence; Ace: Acenaphthene; Fl: Fluorene; Phen: Phenanthrene; Ant: Anthracene; Flu: Fluoranthene; Py: Pyrene; BaA: Benzo(a)anthracene Cr: Crysene; BbF+BjF: Benzo(b)fluoranthene+Benzo(j) fluoranthene; BkF: Benzo(k)fluoranthene; BeP: Benzo(e)pyrene; BaP: Benzo(a)pyrene; BghiPe: Benzo(ghi)perylene; DahA: Dibenzo(a,h) anthracene; InP: Indopyrene.

4. Discussion

As already indicated, the active biomonitoring technique with moss transplants has not been completely standardized, and for example there are some difficulties in interpreting the results of this type of study, often because of the controls used. Couto et al., 2004 proposed determining the LOD and the LOQ from blanks (LOQ_T), because of the variability associated with these. In the present study we therefore used the concentrations at blanks (LOQ_T) and handling controls, the latter of which were subjected to the same processes (laboratory and transportation) as the other samples. This is a methodological innovation as it decreases the possibility of contamination outside the period of exposure, thus increasing the sensitivity of the method and improving the degree of standardization of the active biomonitoring technique.

The results obtained indicate that the study area was not contaminated with As, or with most of the PAHs considered (except fluoranthene and pyrene). Although quantitative treatment of these data may not be valid, because the concentrations of contaminants are higher than the LOQ_T, and they cannot be differentiated from the initial concentrations (at time zero) if the LOQ_T is applied (Couto et al., 2004), they were finally included as the results were consistent. These data should, however be considered with caution. The possibility that the method is not sensitive to the presence of As or certain PAHs can be discounted, as signals were detected for these contaminants in other similar studies (Wegener et al., 1992; Couto et al., 2004; Castello et al., 2007; Aničić et al., 2009).

On the other hand, the highest EFs corresponded to V, with a mean value of 81, and maximum values of up to 200, which appears to indicate serious contamination by this metal in the study area, confirmed by comparison with the data obtained in other European studies. For example, for Belgrade, a city with more than 1,700000 inhabitations, an EF of 24 was obtained in three months and of 58 in six months (Aničić et al., 2009); in other large cities such as Naples (Adamo et al., 2003; Giordano et al., 2005), Sofia (Culicov and Yurukova, 2006) and Budapest (Naszradi et al., 2007) the values were one order of magnitude lower than those obtained in the present study for this element. The mean EFs obtained for Ni were similar to those obtained in Oporto by Vasconcelos et al. (1998) in samples exposed for three months. These levels were three times those obtained in other cities such

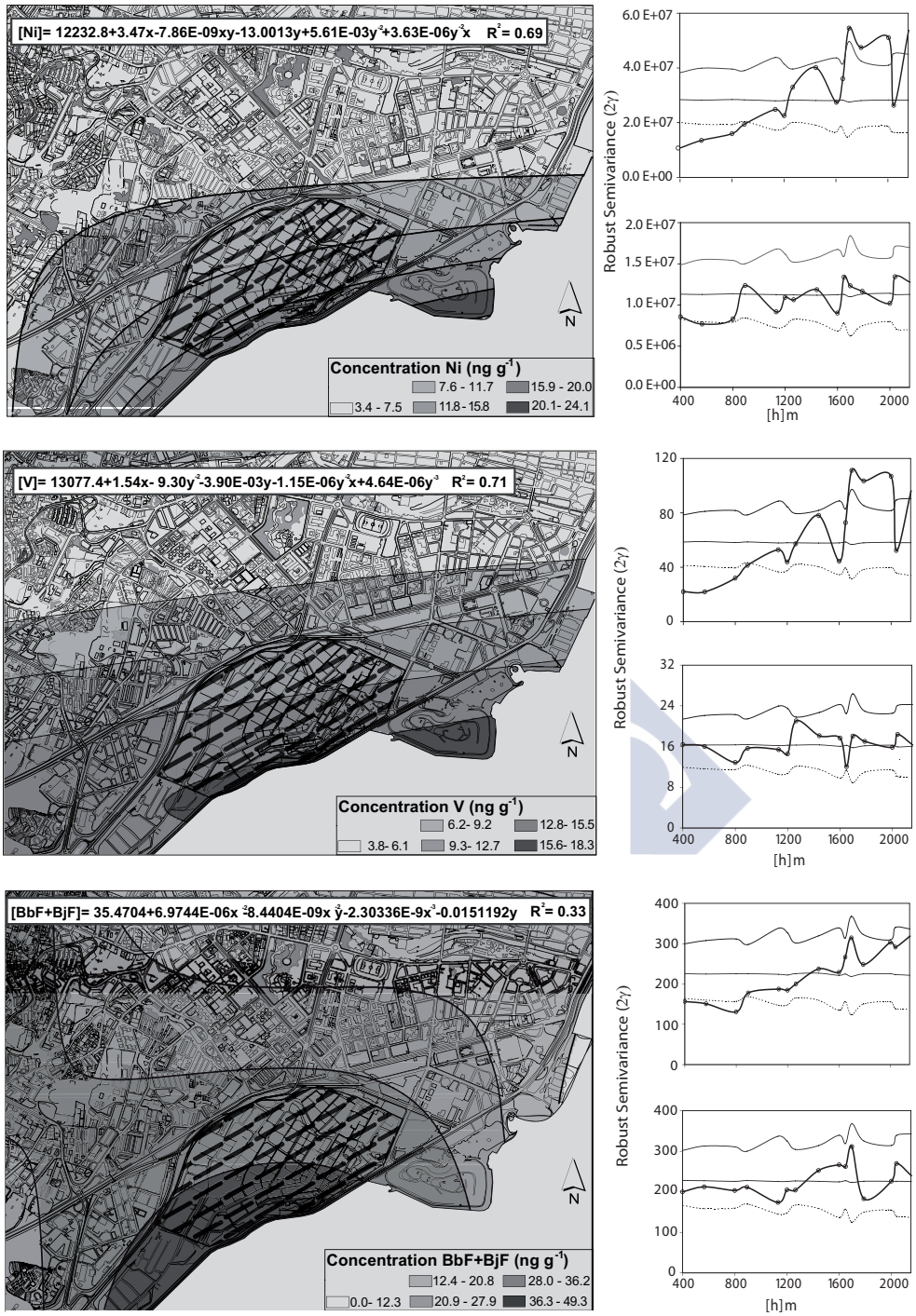


Fig. 4. Representation of the robust semivariograms of the concentrations of Ni, V and benzo(b) fluoranthene+benzo(j)fluoranthene and those carried out for the residuals generated by the response surface in transplants of *Pseudoscleropodium purum* after two months of exposure. The response surface for the spatial structure of these contaminants is also shown, with the polynomial that describes it and the corresponding coefficient of determination. The shaded area indicates the area occupied by the refinery.

as Naples and Trieste, for similar exposure periods (Adamo et al., 2007) and in Belgrade (Aničić et al., 2009) for an exposure period of 6 months. For Pb, the EFs were lower than those for V and Ni, and those obtained in cities such as Oporto (Vasconcelos et al., 1998), Naples (Adamo et al., 2003; Giordano et al., 2005) and Acerra (Basile et al., 2008). The presence of Pb has been associated with a high density of road traffic, which is a serious problem in cities such as Naples, through which estimated 265,000 vehicles travel daily (Giordano et al., 2005). Although an EF of 9 was obtained in Oporto (Vasconcelos et al., 1997), the levels of contamination were much lower than those obtained in studies carried out in previous years in the city, a decrease related to the increasing use of unleaded petrol. In the case of Hg and Cd, the EFs were relatively low and similar to those found in other European studies.

Very few biomonitoring PAHs studies have been carried out with moss transplants. Intensive type studies have mainly concentrated on small scale emission foci, such as: roads with different amounts of traffic (Viskari et al., 1997, Orlinski, 2002), industrialized areas (Wegener et al., 1992) and house interiors (Rantalainen et al., 1999). The EFs obtained in the present study are similar to those obtained for roads in an urban area of Varsovia (Orlinski, 2002) and in a motorway in Finland (Viskari et al., 1997), although lower than those obtained in the surroundings of an aluminium smelter (Wegener et al., 1992). In the present study some extreme values, relative to those reported in the relevant literature and moreover that do not appear to be related to the proximity to the industrial area under study, were obtained. As previously indicated, the active biomonitoring technique with terrestrial mosses is not completely standardized, and the techniques applied to date differ in terms of the species used, the amount of moss in each bag, the shape of the bags and the material that they are made of, and the height that they are situated, among many other variables. Therefore the results cannot be directly compared and are merely guidelines.

An attempt was made to compare the results obtained in the present study with data from contamination measuring station however, PAHs and heavy metals and metalloids were not directly measured in the atmosphere at any sampling sites. Contamination measuring stations are scarce in the area and basically measure the levels of particulate material. PM_{10} and $PM_{2.5}$ were measured at only three sites and one site respectively, during the study period (March – May 2008). The mean levels of PM_{10} for these three sites were 29.45 ± 2.25 μg m^{-3}, 42.36 ± 3.77 μg m^{-3} and 17.15 ± 1.04 μg m^{-3} (mean values ± 95% confidence interval) and those of $PM_{2.5}$ were 10.35 ± 0.52 μg m^{-3} (mean value ± 95% confidence interval), according to the data reported by the Canary Islands Government (http://www.gobiernodecanarias.org /cmayot/ medioambiente / calidadambiental /calidaddelaire/controlyvigilancia/index.html).

Analysis of the chemical composition of these particles was determined only once, a high concentrations of Ni and V were obtained in the Air Quality Plan of the Autonomous Government of the Canary Islands (Technical report, Anonymous 2007). However heavy metals and metalloids (As, Cd, Hg and Pb) and PAHs were not measured in the particulate matter (PM_{10} and $PM_{2.5}$).

Principal components analysis (PCA) has been used in numerous studies as a tool to facilitate interpretation of the relationships between variables and the identification of groups, and a common source identified for various elements (Szczepaniak et al.,

2006, 2007; Sun et al., 2008). The results obtained in the present study, grouped all of the metals and most of the PAHs that displayed spatial structure (i.e. anthracene and crysene) in the second factor, which suggests a common source for these contaminants. In the specific cases of Ni and V, correlation between these elements is often found in moss biomonitoring studies in industrial areas with similar characteristics (Aničić et al., 2009; Čeburnis et al., 1997; Herpin et al., 1996; Tuba and Csintalan, 1993).

Graphic representation of the response surfaces indicates an increasing gradient of concentrations towards the industrial zone where the refinery is, with a certain degree of displacement towards the southeast of the zone. The maps show that the pattern detected is almost identical for Ni and V. Tuba and Csintalan (1993) obtained similar results for the spatial pattern of distribution of Ni and V in a city close to an oil refinery. As the maps elaborated in the present study show, there was also a very sharp decrease in the concentrations of metals with increasing distance from the refinery, especially for the two elements for which the highest enrichment values were obtained (i.e. Ni and V). There was a trend for lower EFs (e.g. for Hg and PAH) to be associated with lower gradients of concentration.

Representation of density distributions for metals and metalloids confirmed the existence of the pattern of dispersal, as almost all of the extreme values belonged to the first 15 points on the grid, situated to the southwest of the refinery. For almost all of the contaminants, there is an area in the northwest where there appears to be a second zone of preferential dispersal of the contaminants. This may be the result of a second source of contamination and/or the orographic characteristics and of the region and/or the type of dispersal of the contaminants, which would be affected by the predominant winds and breezes, the direction of which may modify the gradient of contamination around the emission focus (Gailey et al., 1986; Castello et al., 2007).

The results of the present study have shown that the active biomonitoring technique with moss transplants is suitable for obtaining a detailed assessment of the air quality in the urban and industrialized area of Santa Cruz de Tenerife. Considering that the concentrations of various types of contaminants can be obtained simultaneously, and that a dense sampling network, such as that used in the present study, can be established at a relatively low cost, the moss transplant technique appears ideal for determining the levels of contamination in an area and the corresponding dispersal patterns. The technique may be a useful tool that could be used by the authorities to design environmental monitoring programmes, with resiting of monitoring sites in target zones.

5. Conclusions

The use of the LOQ_T and controls, for the handling and transport of samples, has improved the quality and robustness of the results, and is an important aspect in the standardization of the active biomonitoring of moss transplants technique.

High concentrations and corresponding high enrichment factors were found for Ni and V, consistent with the type of industry considered (an oil refinery). However, no contamination by As or most of the PAHs analysed was detected. The method was found to be suitable for testing for the existence of spatial structure in the concentrations of Ni and V, and also those of Cd, Hg, Pb, with the lowest coefficients of determination obtained for anthracene, crysene and benzo(b)fluoranthene+

benzo(j)fluoranthene.

Representation of the surface response indicates the presence of spatial structure linked to the industrial zone, with slight displacement towards the southwest. The results obtained by application of kernel smoothing supported this interpretation.

Finally, active biomonitoring with terrestrial mosses was found to be an ideal technique for urban and industrial environments, which enabled, easily and economically, the establishment of a dense network of sampling sites and the simultaneous determination of the concentration of heavy metals and PAHs using only one type of sampler. Therefore although this technique has not yet been standardized, it may be used by authorities as a very useful environmental monitoring tecnique.

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General Discussion



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General Discussion

There is a great deal of information available regarding the relationship between atmospheric contamination and human and environmental health. During the last few decades, the different methods involved in biomonitoring atmospheric contamination have been the object of intense study aimed at developing inexpensive tools that would provide representative data on air quality. However, at present none of the tools available is capable of providing wholly reliable data. This applies to both physicochemical methods and to methods based on the use of live organisms. This is mainly due to difficulties associated with atmospheric dynamics as the high spatio-temporal fluctuations in air masses affect the sample representativeness and largely depend on physical agents such as wind and precipitation. Other influencing factors include, on the one hand, the physicochemical characteristics of the contaminants, such as the emission form (gaseous or particulate) and, on the other hand, processes involving the uptake and accumulation of elements in the moss (i.e. physicochemical processes and particle interception) (Varela et al., submitted for publication).

Despite such difficulties, biomonitoring tools are required and must be developed. To date, the "moss bag" technique has predominantly been used in applied studies, mainly in industrial and/or urban areas, with a scarce study of the methodological and theoretical fundaments of the technique. Very few of the studies carried out in the 40-year period in which the technique has been applied, have used the same protocols for preparing and exposing the moss transplants, unless performed by members of the same research group. This has led to some difficulty in comparing the results of different studies. This difficulty is compounded by the fact that the authors of many of the

published studies do not provide a good description of the methods used and simply overlook the possible effects of methodological differences on the interpretation of results. Many of the studies report a certain degree of concordance between the concentrations of contaminants found in the moss and in the study area (i.e. high concentrations in industrialized areas and low concentrations in rural areas); they also often indicate that "moss is a very suitable tool in environmental studies", and some authors even assure that moss bags can replace more complicated, lengthy and cost consuming tools (Szczepaniak et al., 2007).

The main aims of the present study were therefore to investigate and optimize the key methodological steps in the "moss bag" technique and to propose a standard method for the correct application of this tool. This will help us understand the relationship between the concentration of contaminants in the atmosphere and in the moss tissues. This detailed research will also provide a realistic view of the limits of the technique.

The literature review (State of the art) has highlighted the methodological gaps in the protocol, and 18 variables involved in the "moss bag" technique were identified. These aspects comprise some of the key steps in the protocol (i.e. selection and preparation of moss, preparation of bags, exposure conditions and post-exposure treatments) and therefore must be optimized before systematic application of the technique is possible. The variables may interact with each other, and therefore an iterative optimization procedure, in which one variable is modified while the others are held constant, is recommended. This procedure should be carried out in future research. As iterative optimization is a lengthy procedure, only 5 steps were able to be optimized in the present study (described in Chapters I and II): pre-exposure treatment, selection of species, the relationship between moss weight

and bag surface area, the duration of exposure and the height of exposure. The optimal mesh size of the material used to make the bags has been established by other authors (Giordano et al., 2013). Nonetheless, numerous aspects must still be optimized under different field conditions.

Another requirement of an effective biomonitoring tool is that there should exist a linear relationship between the atmospheric concentrations of the elements under consideration and the concentrations in moss. In studies carried out to date, including the study described in Chapter III, the concentrations of heavy metals in moss have only been compared with those in the soluble fraction of the bulk deposition, and the particulate fraction has been overlooked. The relationship between the concentration of the particulate fraction of the bulk deposition and the concentration in the moss tissue must therefore be tested. Likewise, the correlation with dry deposition must also be tested (also for both the gaseous and particulate fractions).

Clearly, investigation of many aspects of the technique is still required. Such studies are currently underway as part of the MossClone project (Seventh Framework Programme, Research Consortium, European Commission), in which several European research groups involved in biomonitoring and related research are participating, with the aim of optimizing and validating the technique. The main methodological and theoretical problems being addressed in the project are as follows:

- Research involving the influence of methodological aspects such as the shape of the moss bags, which may affect the levels of pollutants adsorbed by the moss in terms of replicability and data quality. Three bag shapes are being studied: flat, semispherical and spherical, the latter only allowing a single layer of moss.
- Chapters I and II have highlighted some problems inherent to the

use of native mosses for transplanting: availability of the species in the study area, difficulties in identifying the species in the field, and the natural variability (both local and temporal) in the contaminants accumulated in the moss (Boquete et al., 2011; Couto et al, 2004). Laboratory cultivation of a moss clone would solve these problems. This may soon be possible as a stable culture of *Sphagnum palustre* L. has recently been established. The availability of cloned moss would also enable chemical and morphological characterization by molecular techniques (DNA fingerprinting, etc.), chemical composition (multi-elemental analysis), and physical and physical-chemical characterization (e.g. surface stability constants, specific surface area, maximal surface adsorption capacity, physical heterogeneity, porosity, surface charge, etc.) (González and Pokrovsky, 2014). This would yield a highly standardized biomonitoring tool.

The MossClone project also aims to study the theoretical bases of the technique in more detail, specifically to test the validity of the technique in comparison with other traditional systems of atmospheric contamination monitoring, such as particle samplers, gaseous samplers and passive samplers. The fact that the concentrations of particulate material in moss are related to the concentrations in air is of particular interest as although some atmospheric contaminants, including heavy metals, may be present in gaseous form in the air, most of them are associated with particles in suspension (Bargagli, 1998). Particulate material comprises many particles of different sizes (Spagnuolo et al., 2013), which can be respired and ultimately incorporated in the respiratory apparatus of living organisms. Indeed, moss can trap very heterogeneous and complex particulate material, and the occurrence of certain particulate forms is highly informative about the mechanisms of uptake in moss and in its environment (Adamo et al.,

2007). It has been estimated that moss can intercept 98% of PM_{10} particles (Tretiach et al., 2011).

The standardization of moss bags for monitoring air quality is not an isolated project, and several other similar projects are currently underway. For example, the M.O.S.S.B.A.G.S. project (Italian Environmental protection agencies- University of Trieste), also aims to standardize the protocols for the field collection and laboratory processing of aquatic mosses for monitoring the concentrations of contaminants in surface waters. Regarding the passive biomonitoring of air quality, the European Committee for Standardization has published a standard method (CEN/TC 264/WG 31: Biomonitoring methods with mosses and lichens); this initiative will allow background levels of pollutants to be mapped at national or global levels via the use of moss samples, and the establishment of a biological index of epiphytic lichens will enable assessment of the biological effects of air quality on ecosystems.

The high variability in the results of the "moss bag" technique may, a priori, represent an important restriction to the application of the technique by the relevant authorities. However, traditional methods also yield variable results. Aas et al. (2009) reported low reproducibility of data obtained with collectors used to estimate trace elements in wet and dry deposition. Nonetheless, this method is accepted under current legislation and is widely used throughout the European Union (The European Monitoring and Evaluation Programme, EMEP). However, the "moss bag" technique still cannot be used to provide quantitative results. As a result of methodological-type studies, such as that presented here, or those being developed in projects such as MossClone, the variability in the data will diminish; moreover, within the complex task of atmospheric biomonitoring, it has been shown that the technique can provide valuable qualitative information about the atmosphere in certain areas and that the technique can be used

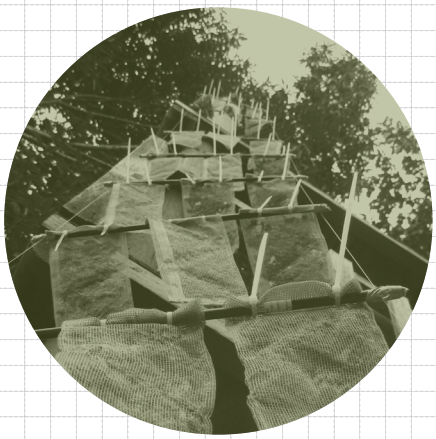
to complement traditional methods. For example, significant correlations between the concentrations of Cd in moss samples and in bulk deposition have often been found, as in this research (Chapter III). A good correlation has also been highlighted for Pb and Hg in different studies (Aboal et al., 2010). One of the most plausible theories in this respect is that these three elements have a large ionic radius and large covalent bond index, and they are therefore absorbed more efficiently than other elements onto particles. Previous studies have demonstrated that moss bags retain such particles efficiently (Spagnuolo et al., 2013; Tretiach et al., 2011).

Qualitative use of the technique has been emphasized by many authors, and some even indicate that use of these bioaccumulators is often the only possible approach for constructing maps of airborne pollutants in urban and remote areas (Sardanas and Peñuelas, 2005). Chapter IV outlines the usefulness of the "moss bag" technique for this type of study; the establishment of a standard method would enable comparison of other similar studies, thus enable mapping of larger areas and integration of the information obtained. This approach would enable, e.g., mapping of the spatio-temporal distribution of contaminants throughout Europe.

The technique may also be particularly useful as a tool for diagnosing air quality, to provide a preliminary estimation of the environmental status of an area. This would enable an environmental policy to be established as a starting point for improved economic management of the available resources and to prioritize the location of these devices in identified contamination "hotspots". The use of the "moss bag" technique may contribute directly or indirectly to a reduction in the use of materials, resources, energy consumption, contaminant emissions, etc., and it has both economic and ecological benefits.

However, the real value of the data and the limits of active biomonitoring techniques must always be borne in mind.

General Conclusions



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General Conclusions

- The vitality of the exposed moss samples has an important effect on uptake of heavy metals by moss and uptake is not exclusively passive or only based on surface interception. As a result of active metabolic processes, some metals may be mobilized to apical portions of the moss shoots (i.e. Cd, Cu and Zn) and others towards basal parts (i.e. Hg and V), leading to underestimation or overestimation of the concentrations. Therefore, the following are recommended:

  - The use of devitalized moss for biomonitoring studies of air quality.

  - Use of live moss (with an irrigation and shading system) only when the objectives of the study include analysis of physiological variables and taking into account the effect of growth for correct analysis of the results.

- Optimization of the most variable aspects of the methodology was carried out on the basis of the concentration "signal" detected and the degree of replicability of the signal. The most important findings in this respects were as follows: that the most appropriate species of moss is *Sphagnum denticulatum*, the optimal quantity of moss per surface area of bag is 5.68 mg cm^{-2}, an exposure period of 8 weeks is a compromise between the signal detected and the replicability, and as a result of the lack of any clear findings related to the height of exposure, a height of 4 m is recommended for mainly practical reasons.

- Moreover, on applying the technique, the height and duration of exposure are the aspects that introduce the greatest degree of variability in the results. Use of the established protocol is therefore

particularly recommended to minimize the variability in these aspects.

- The general lack of standardization of the moss bag technique hampers routine use of the technique as a regular tool for environmental monitoring by official institutions. Taking into account the results of studies carried out during years of application of the technique, especially those studies that focus on the methodology and those carried out for this doctoral research, an optimized protocol for use of the technique is proposed:

Variable	Recommendations
1. Preparation of the moss	
1.1. Selection of species	*Sphagnum sp*[e] preferably from a cloned culture.
1.2. Selection of material	Green apical portions (5 cm)
1.3. Pre-exposure treatments and vital state	Devitalized
1.3.1. Washing with cellular extractants	
1.3.1.1. Number of washes	1 wash with EDTA (10 mM) + 1 with Dimercaprol (30 mM)
1.3.1.2. Duration of washing	20 minutes
1.3.1.3. Shaking	Wash with shaking
1.3.1.4. Weight of moss per volume of extractant	1 L water per 12 g dw
1.3.2. Washing with water	Water washed
1.3.2.1. Number of washes	3 times
1.3.2.2. Duration of washing	20 minutes
1.3.2.3. Shaking	Wash with shaking
1.3.2.4. Type of water	Distilled water
1.3.2.5. Weight of moss per volume of water	10 L water per 100 g dw
1.3.3. Devitalizing treatment	Oven drying, 24 h 120° C[a,b]
2. Preparation of transplants	
2.1. Mesh material	Nylon mesh
2.2. Mesh size	2 mm[d]
2.3. Shape of bag	Spherical[c] (ensuring single layer of moss)
2.4. Size of bag	12 mg cm^{-2} [e]
3. Exposure	
3.1. Shading system	No conclusion
3.2. Cover	No conclusion
3.3. Location and type of support used	Free of obstacles, suspended from inert support by nylon thread
3.4. Height of exposure	4 m[e]
3.5. Duration of exposure	8 weeks[e]
3.6. Number of bags for site	3 bags
3.7. Initial concentrations and controls	3 controls and 3 initial times
4. Post exposure treatment	No treatment

[a] Fernández et al. (2010); [b] Giordano et al. (2009); [c] Gailey and Lloyd (1986a); [d] Giordano et al. (2013); [e] present study

- Most of the significant correlations among concentration levels in moss and bulk deposition involved Cd and to a lesser extent Cu and Zn. The environmental conditions and particularly the abundance of precipitation are the main causes of the absence of correlations in some cases, presumably because of the loss of elements by leaching. In according with this, most of the significant correlations were observed in the driest months. The type and amount of emission seems to have less importance in relation to the presence of correlations between the elements under study. The total lack of correlations for Pb and Hg indicates the need for further studies to determine the relationship between the concentration of trace elements in moss bags and the particulate fraction in the bulk deposition.

- It has been demonstrated that the moss bag technique is an ideal technique for biomonitoring urban and industrial environments. Its use has enabled the establishment of a dense network of sampling sites and the simultaneous determination of the concentration of different type of contaminants by using only one type of sampler. This shows that the technique is suitable as a diagnostic tool for measuring air quality.

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General References



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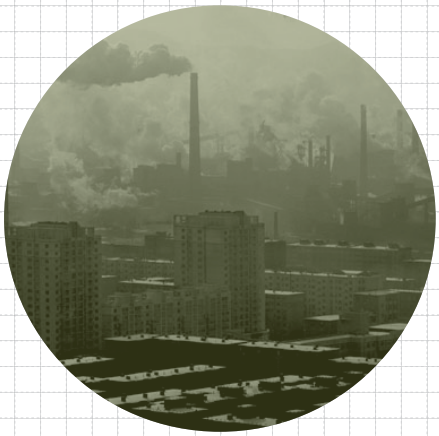
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Summary (Resumen)



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Summary (Resumen)

La contaminación atmosférica es uno de los problemas más críticos que actualmente afectan a nivel mundial. Las emisiones contaminantes han ido en creciente aumento desde la Revolución Industrial, acentuándose notablemente desde la Segunda Guerra Mundial como consecuencia del importante desarrollo tecnológico. Pese a que la calidad de vida ha mejorado notablemente desde entonces, La presencia en el ambiente de sustancias contaminantes como los metales pesados puede producir efectos nocivos graves e incluso irreversibles sobre la salud humana y los ecosistemas, tal y como reconocen diversas agencias internacionales como la Environmental Protection Agency de los Estados Unidos (USEPA) o la Agency for Toxic Substances and Disease Registry (ATSDR). Por esta razón se han venido promulgado una serie de directivas, con el fin de intensificar la evaluación y control de la calidad del aire ambiente referidas a la los metales pesados. A nivel europeo, Directivas como la 1999/30/EC y la 2004/107/EC obligan a la monitorización periódicas de la deposición atmosférica de metales como Pb, Cd, As y Ni.

Sin embargo, la monitorización de los contaminantes en el aire es una tarea muy complicada y las técnicas de control de la contaminación atmosférica han sido objeto de una intensa investigación durante las últimas décadas. Estas medidas se llevan a cabo habitualmente mediante la aplicación de métodos físico-químicos para cuantificar los niveles de los contaminantes en la atmósfera. Estos métodos pueden proporcionar medidas precisas de algunos contaminantes atmosféricos principales (mayoritariamente CO, SO_x, N_xO_y, PAHs y material particulado) y en periodos relativamente cortos de tiempo (i.e. horas o días). Sin embargo determinar directamente los metales pesados mediante el empleo de éstas técnicas presenta

mayores dificultades.

Una alternativa en el control de dichos contaminantes es el empleo de seres vivos como biomonitores, destacando entre todos ellos los musgos terrestres. Las ventajas de éstos comparados con las técnicas físico-químicas son su simplicidad, su coste y la falta de requerimiento de energía eléctrica. Los musgos terrestres poseen además una serie de características físico-quimicas y morfológicas (Bargagli, 1998) que les confieren una alta capacidad de intercambio catiónico y una morfología adecuada para fijar y acumular contaminantes presentes tanto en forma gaseosa como particulada, y de naturaleza tanto orgánica como inorgánica, procedentes de la deposición atmosférica. En la literatura científica sobre la biomonitorización de la contaminación atmosférica mediante musgos, se diferencian de forma clara dos opciones a la hora de emplear estos organismos: (i) biomonitorización pasiva, recogiendo musgo que de manera natural crece en una determinada zona y (ii) activa, trasplantando musgo procedente de otras localizaciones. Esta última forma consiste en recolectar musgo de zonas relativamente limpias, seleccionar el material vegetal que será trasplantado, aplicar diferentes tipos de tratamientos pre-exposición y exponer los trasplantes en un entorno diferente. La utilización de los trasplantes resuelve diversos problemas que presenta el empleo de musgo nativo, como son: (i) la escasez o ausencia de musgo en ciertos ambientes (i.e. áreas industriales y urbanas); (ii) la elevada heterogeneidad en las cargas contaminantes que presenta el musgo dentro de una misma estación de muestreo (Aboal et al., 2006; Fernández et al., 2002); (iii) la interpretación de los resultados obtenidos respecto al tiempo, ya que en los trasplantes la concentración inicial y el período de exposición es conocido (iv) la posible influencia edáfica en las concentraciones de ciertos elementos (ver por ejemplo: Bargagli, 1995; Berg and Steinnes, 1997); (v) las

adaptaciones fenotípicas y/o genotípicas que pueden producirse en ambientes contaminados (Fernández et al., 2000; Tabors et al., 2004), dicho problema se solucionaría trasplantando musgo de zonas no contaminadas. Además, la influencia de los contaminantes en la fisiología en el musgo nativo es difícil de evaluar puesto que no es posible aislar su efecto de otras variables ambientales. Sin embargo, con el empleo de trasplantes irrigados es posible eliminar ciertos factores de estrés ambiental (e.g. estrés hídrico o por radiación solar) aislando el efecto de la contaminación.

Dentro de los diferentes tipos de trasplantes de musgo, la técnica de las "moss bags" es el tipo de trasplante más común. Fue empleado por primera vez por Goodman and Roberts (1971) y consiste en la exposición de muestras de musgo contenidas en bolsas de malla. En la bibliografía consultada (aproximadamente 120 trabajos publicados entre 1971 y 2014, localizados mediante SCOPUS) se comprueba que la técnica se emplea principalmente para controlar los niveles de contaminantes inorgánicos, fundamentalmente metales pesados (86%) y, en menor medida, de contaminantes orgánicos PAHs (4%) y PCBs (1%). De entre los metales pesados, destaca el Pb que ha sido determinado en un 62% de los trabajos, seguido del Zn (51%), Cu (48%), Cd (45%), Fe (43%) y Ni (40%).

Sin embargo, a pesar de los 40 años en los que se llevan empleando las "moss bags", todavía no se dispone de protocolos standard de aplicación de la técnica. Éstos deberían de incluir aspectos relacionados con: (i) la preparación del musgo; (ii) preparación de los trasplantes; (iii) la exposición de éstos y (iv) el tratamiento postexposición. Hoy en día existen numerosos protocolos diferentes, que se traducen en una importante falta de estandarización de la técnica que limita la interpretación de los resultados y dificulta la comparación de las conclusiones obtenidas en diferentes investigaciones.

La consecuencia de todo esto es que, a pesar de las ventajas de la técnica, su uso se encuentra restringido exclusivamente al ámbito de la investigación científica, sin que hasta el momento, se haya implementado su uso en el control de la contaminación atmosférica por parte de las administraciones responsables.

Otro de los requisitos clave en la técnica de la biomonitorización, para que su uso sea considerado como una alternativa válida a los métodos tradicionales, es que las concentraciones halladas en el organismo biomonitor deben reflejar adecuadamente los niveles atmosféricos. En el caso de la biomonitorización pasiva con musgos nativos se han encontrado correlaciones significativas especialmente para Cd, Hg y Pb y en menor medida en Cu y Zn (Aboal et al., 2010). Mientras que la bibliografía disponible sobre la técnica de las "moss bags" es más limitada y muchos de los trabajos han sido llevados a cabo siguiendo protocolos muy diferentes.

Objetivos generales

Optimización de aspectos metodológicos claves en la aplicación de la moss bag technique mediante:

La evaluación cuantitativa del efecto de la vitalidad del musgo, mediante el estudio del efecto del crecimiento en la carga de metales pesados durante el período de exposición. Para dicho fin se emplean trasplantes autoirrigados y sombreados ya que permite mantener constante la fisiología del musgo.

La selección de la opción óptima para diferentes aspectos metodológicos: i) selección de la especie, ii) la relación entre el peso de musgo empleado y el area superficial de la bolsa, iii) la duración de la exposición and iv) the altura de la exposición en función de los valores de replicabilidad y la concentración final de metales pesados

en el musgo.

Investigación de la existencia de correlacion entre los niveles de contaminantes encontrados en el musgo en relación a la deposición total. Mediante el empleo de "moss bags" desvitalizadas en lugares con diferentes grados de contaminación y mediante diferentes períodos de exposición.

Validación de la técnica mediante el estudio de los patrones de dispersión espaciales de contaminantes (metales pesados y PAHs) en un área industrial, urbana y con elevada densidad de tráfico rodado.

Objetivos principales, resultados y conclusiones de los diferentes capítulos de esta tesis

Capítulo 1. Efecto del crecimiento en biomonitorización activa con musgos terrestres.

Los efectos del crecimiento en trasplantes sombreados y autoirrigados de *Pseudoscleropodium purum* en la cuantificación de las concentraciones en los tejidos de Cd, Cu, Hg, V y Zn, fueron investigados en 4 períodos de exposición, cada período de 56 días, en 7 puntos de muestreo (contaminado y no contaminada). Las concentraciones de los elementos en las porciones basales de los tallos se compararon con las concentraciones en las partes de los tallos que crecieron durante el período de exposición. Se encontraron concentraciones más bajas de Hg y V en las nuevas porciones de los tallos que en las porciones basales, mientras que ocurría lo contrario para Cd, Cu y Zn. La magnitud de error introducido por el crecimiento no se puede ignorar, siendo en algunos casos era mayor que el 40%, con relación a los resultados obtenidos por análisis de todo el tallo. Se recomienda por tanto la desvitalización de musgo antes de su uso como material

de trasplante para evitar el efecto del crecimiento de la planta durante el período de exposición.

Capítulo 2. Hacia una optimización metodológica de la técnica de "moss bag" en cuanto a concentración contaminante y valores de replicabilidad.

La técnica de las "moss bags" es una herramienta simple y económica de vigilancia ambiental se utiliza comúnmente con el fin de monitorizar la calidad del aire. Sin embargo, el uso rutinario de este método no es todavía posible ya que los protocolos de elaboración y exposición de los trasplantes en el área de estudio aún no se han estandarizado. Algunos de los aspectos metodológicos más variables incluyen: (i) la selección de la especies de musgo, (ii) la relación existente entre el peso de musgo por unidad de superficie de la bolsa, (iii) la duración de la exposición, y (iv) la altura de la exposición. En el presente estudio, se seleccionó la mejor opción para cada uno de estos aspectos en base a dos criterios, la concentración media y los datos de replicabilidad de Cd, Cu, Hg, Pb y Zn. El experimento se llevó a cabo durante al menos dos periodos de exposición en ambientes afectados por diferentes grados de contaminación. Los resultados indicaron que las opciones óptimas para los aspectos estudiados fueron: (i) *Sphagnum denticulatum*, (ii) 5.68 mg de musgo tejido para cada cm^2 de superficie de la bolsa, (iii) de 8 semanas de la exposición, y (iv) 4 m de altura de la exposición. La duración y la altura de exposición resultaron ser los aspectos metodológicos que mayor variabilidad aportaron en los datos. El resultado de este estudio metodológico permite dar un paso más en la estadarización de la técnica y que permita el uso de las "moss bags" por parte de las autoridades públicas.

Capítulo 3. Biomonitorizacion de la calidad del aire empleando

***Sphagnum denticulatum* "moss bags": Una comparación con la deposición total**

Los elementos traza en la deposición atmosférica han sido siempre una preocupación para la salud ambiental y humana. Lo más común es el empleo de métodos tradicionales para monitorizar sus concentraciones aunque el uso de la técnica de "moss bags" supone un método más simple y económico. Sin embargo todavía no se comprenden algunos aspectos teóricos de esta última, como la relación entre los niveles de metales pesados acumulados en el musgo y las concentraciones de éstos en la atmósfera. En el presente estudio, se determinó la correlación entre la concentración de elementos traza (Cd , Cu , Hg , Pb y Zn) en bolsas de musgo desvitalizado *Sphagnum denticulatum* y la deposición total, durante 12 períodos de exposición en 21 estaciones de muestreo afectados por diferentes grados de contaminación. La mayoría de las correlaciones significativas se encontraron para el Cd y en menor medida de Cu y Zn , sin embargo no se encontraron correlaciones para Pb y Hg . Las condiciones ambientales y particularmente la abundancia de precipitación son las principales causas de la ausencia de correlaciones en algunos casos, presumiblemente debido a la pérdida de elementos por lixiviación. La técnica de las "moss bags" es una herramienta ambiental económica muy útil teniendo siempre en cuenta el valor real de los datos y los límites de la técnica.

Capítulo 4. Estudio de la calidad del aire en áreas industriales de Santa Cruz de Tenerife (España) mediante biomonitorización activa con *Pseudoscleropodium purum*.

En este trabajo se ha empleado la técnica de las "moss bags" con musgos terrestres en un área de Santa Cruz de Tenerife próxima a una

refinería de petróleo y a la zona de mayor densidad de tráfico rodado, mediante la implantación de una malla de muestreo compuesta por 50 estaciones de muestreo y situados a una distancia de 400 m. La exposición se llevó a cabo durante dos meses. Además de la representación de la distribuciones de densidad, del cálculo de los factores de enriquecimiento y del análisis multifactorial se han elaborado mapas de contaminación de la bioconcentración obtenida y tras comprobar la existencia de estructura espacial, se han representado las superficies de respuesta para cada uno de metales y metaloides (As, Cd, Hg, Ni, Pb, V) e hidrocarburos aromáticos policíclicos (PAH). Los resultados indican que la zona de estudio tiene niveles de contaminación muy elevados para Ni y V, los cuales presentan un patrón de dispersión similar. Para Cd, Hg y Pb las concentraciones encontradas son menores y en el caso del As todos los datos obtenidos son menores al límite de cuantificación (LOQ) por lo que no parece existir contaminación por dicho metal. En cuanto a los PAH, salvo fluoranteno y pireno, muchos de los valores obtenidos son menores al LOQ. Se ha demostrado que la biomonitorización activa con musgos terrestres es una técnica apropiada para establecer programas de vigilancia ambiental a bajo coste en entornos urbanos e industriales.

Conclusiones generales

1. La vitalidad del musgo que se emplea en los trasplantes tiene un efecto determinante en el proceso de carga de los metales pesados concluyéndose que este proceso no es exclusivamente pasivo o únicamente basado en la intercepción superficial. Esto puede deberse a que durante el período de exposición, debido al metabolismo activo del musgo, algunos metales pueden movilizarse hacia las partes apicales (i.e. Cd, Cu and Zn) y otros hacia las partes basales (i.e. Hg

and V) dando lugar a una infraestimación o sobrestimación de las concentraciones. Por tanto se recomienda:

- El empleo de musgo desvitalizado en caso de llevarse a cabo estudios de biomonitorizacion activa de la calidad del aire.
- El empleo de musgo vivo (con un sistema de irrigación y sombreados) únicamente únicamente cuando el objetivo del studio incluya el análisis de las variables fisiológicas teniendo presente el efecto del crecimiento para el correcto análisis de los resultados.

2. Las optimizaciones de los aspectos metodológicos con mayor variabilidad en la bibliografía se efectuaron en base a la señal de concentración detectada y su grado de replicabilidad. De esta manera se determinó: que la especie de musgo más apropiada es *Sphagnum denticulatum*, que 12 mg cm^{-2} es la cantidad óptima de musgo por superficie de la bolsa, que 8 semanas de exposición es una compromiso entre la señal detectada y la replicabilidad y que, debido a la falta de patrón existente en la altura de exposición, se determinaron 4 m de altura teniendo en cuenta consideraciones prácticas.

Además en la aplicación de la técnica, la altura y la duracion de la exposicion son los aspectos que introducen mayor variabilidad en los resultados. El empleo de un protocolo establecido es por tanto especialmente recomendado conforme a estos aspectos con el fin de minimizar la variabilidad.

3. La falta general de estandarización de la técnica de las "moss bag" impide su uso rutinario de la herrramienta para la monitorización de la calidad del aire por parte de las instituciones oficiales. Teniendo en cuenta toda la bibliografía disponible sobre el tema, especialmente los estudios más enfocados a la metodología y los realizados durante la presente tesis se ofrece un protocolo optimizado de aplicación de la técnica.

4. En el marco teórico de la técnica de las "moss bags", el presente trabajo ha permitido dar un paso más en el conocimiento del tipo de relación existente entre las concentraciones de los contaminantes presentes en el musgo y las presentes en la fracción soluble de la bulk deposition.

La mayor parte de las correlaciones significativas fueron encontradas para Cd, y en menor medida para Cu y Zn. Los resultados muestran que las condiciones ambientales y especialmente la abundancia en la precipitación, son los causantes de la falta de correlación presumiblemente al provocar la pérdida de elementos por efecto del lavado, además el mayor número de correlaciones están relacionadas con los meses más secos. Por otro lado parece que la naturaleza de la emisión y su cantidad tiene menor influencia en la presencia de correlación entre los elementos estudiados. La falta total de correlacion para el Pb y Hg en todos los casos estudiados indica que es necesario llevar a cabo futuras investigaciones para determinar el grado de relación entre la concentración de elementos traza en las " moss bags" y la fracción particulada de la bulk deposition.

5. Se ha demostrado que la técnica de las "moss bags" es una técnica idónea para ser aplicada en entornos industriales y urbanos como herramientade diagnóstico de la calidad del aire, ya que permite de forma fácil y económica, el establecimiento de una densa red de estaciones de muestreo y la determinación simultánea de de la concentración de diferentes tipos de contaminantes en una sola muestra.

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Estado actual de la técnica y futuras investigaciones

La realidad de la monitorización de los metales pesados en el aire ambiente es que día de hoy ninguna herramienta es capaz de obtener datos representativos del área de estudio y que sea fiable al 100%. Y esto sucede tanto en métodos físico-químicos como en métodos basados en el empleo de organismos vivos. Ello se debe principalmente a las dificultades asociadas a la dinámica atmosférica ya que las altas fluctuaciones espacio-temporales de las masas de aire complican la representatividad de las muestras, altamente condicionada por agentes físicos como el viento y la precipitación. Además influyen también otros factores: por un lado, las características fisicoquímicas de los contaminantes, en las que intervienen aspectos propios de la emisión relacionados con la cantidad y/o la forma de emisión de los contaminantes (i.e. gaseosa/particulada) y por otro lado, en el caso del musgo, también intervienen procesos relacionados con los mecanismos de acumulación en sus tejidos (i.e. procesos fisicoquímicos, e intercepción de partículas) (Varela et al., 2014).

En la presente Tesis se ha avanzado por un lado en el estudio de las implicaciones metodológicas de la técnica de las "moss bags" optimizando la técnica mediante la propuesta de un standard para la correcta aplicación de esta herramienta y, por otro lado en comprender mejor el tipo de relación existente entre los niveles contaminantes atmosféricos y los encontrados en el musgo.

Sin embargo el número de frentes abiertos en la técnica todavía es muy elevado. Actualmente la continuación de estos estudios es llevada a cabo mediante el proyecto MossClone (Seventh Framework Programme, Research Consortium, European Comission) en el que participan varios grupos de investigación europeos del área de la

biomonitorización y otras áreas afines con el propósito de optimizar y validar la técnica. Por lo que algunos de los problemas metodológicos y teóricos principales están siendo investigados en este proyecto, como son:

- La continuación de investigaciones relacionadas con la influencia de aspectos metodológicos como: la forma de las "moss bags".
- Se ha conseguido establecer de forma eficiente un cultivo estable de *Sphagnum palustre* L. de esta forma se puede obtener una herramienta altamente estandarizada y siempre disponible en el laboratorio. El hecho de disponer de un clon de musgo, permite además la caracterización tanto química como morfológica mediante caracterización molecular, composición química o caracterización físicoquímica (por ejemplo la constante de la estabilidad superficial, la capacidad máxima de adsorción superficial, porosidad, etc.) (González and Pokrovsky, 2014).
- Por otro lado, el proyecto MossClone pretende estudiar mejor los fundamentos teóricos de la técnica, concretamente comprobando su validez mediante la comparación con otro tipo de dispositivos tradicionales monitores de la contaminación atmosférica como los captadores de partículas y/o gases.

La estandarización de las "moss bags" para el control de la calidad del aire, no es un hecho aislado, actualmente también se encuentran en marcha otros proyectos para la estandarización de bio-herramientas similares. Un ejemplo es el caso del proyecto M.O.S.S.B.A.G.S. (Italian Environmental protection agencies- University of Trieste) cuyo objetivo es también la estandarización de los protocolos de recogida tanto en campo como de procesado de las muestras en el laboratorio de musgo acuático para monitorizar las concentraciones contaminantes en aguas superficiales. Por otro lado, en el caso de la

biomonitorización pasiva de la calidad del aire, existe actualmente una norma en el Comité Europeo para la Estandarización (CEN/TC 264/WG 31: Biomonitoring methods with mosses and lichens).

A priori, la elevada variabilidad encontrada en los resultados de la técnica de las "moss bags" podría suponer una importante limitación en la aplicación de la técnica por parte de las autoridades. Sin embargo, la presencia de variabilidad en los resultados, también existe en métodos tradicionales tal y como se ha demostrado en el Capítulo III. Aas et al. (2009) afirman que los colectores empleados en estimar las concentraciones de metales pesados en deposición húmeda y seca presentan todavía una reproducibilidad muy baja. Y sin embargo ésta es la metodología aceptada por la legislación siendo su uso muy extendido en la comunidad europea (The European Monitoring and Evaluation Programme, EMEP). La realidad es que a día de hoy, la técnica de las "moss bags" sigue sin poder ser empleada de forma cuantitativa. Gracias a los estudios de tipo metodológicos, como los presentados en esta tesis, o los que están siendo desarrollados por proyectos como MossClone hacen que parte de la variabilidad en los datos se puede disminuir. La técnica puede proporcionar una información muy valiosa sobre el estado de la calidad del aire en un área determinada de una forma cualitativa, que pueda servir como metodología complementaria a los métodos tradicionales. La utilidad de la técnica de una forma cualitativa ha sido destacada por diversos autores, algunos, incluso señalan que el empleo de estos biomonitores es a menudo la única forma posible de elaborar mapas de contaminantes del aire tanto en zonas urbanas como rurales. (Sardanas and Peñuelas, 2005). En la introducción de esta Tesis ha quedado demostrado la utilidad de la técnica de las "moss bags" para esta aproximación, el establecimiento de un standard, permitiría hacer estudios similares que sean completamente comparables, que per-

mitan mapear áreas de mayor tamaño y unificar la información. Mediante este tipo de aproximaciones sería posible, por ejemplo disponer de mapas de distribución espaciotemporal de los contaminantes en toda Europa.

Además puede ser especialmente útil como herramienta de diagnóstico de la calidad del aire, de esta forma se podría disponer de una primera aproximación de la situación ambiental de un área que permita definir una correcta política ambiental como punto de arranque para gestionar económicamente mejor los recursos disponibles y priorizar la colocación de estos dispositivos en los identificados "hotspots" de contaminación. El empleo de las "moss bags" puede contribuir directa o indirectamente a la reducción del uso de materiales, recursos, consumo de energía, emisiones contaminantes, etcétera, presentando ventajas tanto económicas como ecológicas.

Sin embargo es necesario tener siempre presente el valor real de los datos y los límites de la biomonitorización activa.

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