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Mosses for monitoring air pollution: towards the standardization of *moss-bag* technique and the set-up of a new biomaterial

Ph. D. dissertation by

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The objectives and results of this Doctoral Thesis were obtained by research work conducted in the framework of two projects, the EU FP7 Mossclone and the LIFE11/ENV/IT/275 ECOREMED, which have financed my PhD, and in particular by collaboration with dr. Oleg Pokrovsky of the GET-CNRS (Toulouse, France) where I spent part of my PhD period.

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ABSTRACT

The monitoring of the air quality by mosses provides qualitative and quantitative data using economic, easy-to-manage and eco-friendly methods. Due to peculiar morphological and physiological characteristics, mosses are very suitable adsorbents for a wide variety of pollutants (i.e. metals and metalloids, PAHs, radionuclides) and, when used as transplants in nylon bags, they allow an easy monitoring potentially of any site, with a highly dense sampling network.

Nevertheless, the moss-bag technique, although widely applied, is still not based on standardized protocols. It follows that the data collected from biomonitoring surveys are not comparable, thus relegating the active biomonitoring exclusively to academic field or to scientific purposes. Moreover, there are not enough studies comparing bioaccumulation data with those obtained by estimation models or traditional monitoring approaches, neither it is clear how the moss-bag technique can discriminate pollution inputs on a very small scale and within areas relatively close to each other or characterized by different land uses. Another important issue is that the mosses employed as biomonitors are naturally grown species. The collection in nature implies an intrinsic variability of mosses in terms of elemental and chemical composition and, as a consequence, it poses a high degree of uncertainty in the interpretation of the results. Moreover, an uncontrolled harvesting of mosses could lead to a severe environmental impact.

In this context, aims of this Doctoral Thesis are: 1) to test the variables affecting the exposure protocol in the view of a standard moss-bag method for the biomonitoring of air pollution; 2) to integrate biomonitoring results with emission data provided by inventories for the evaluation of the atmospheric pollution; 3) to characterize a novel moss biomaterial for biomonitoring purposes.

For the standardization assay, more than one thousand moss bags were exposed contemporary in three European territories (Austria, Italy, Spain) belonging to three different climatic areas (Mediterranean, continental and oceanic). For each area, four distinct scenarios (background, urban, agricultural, industrial) were selected for the exposure, on the basis of their level and type of contamination. The moss included in bags was the *Pseudoscleropodium purum* (Hedw.) M. Fleisch, collected in a pristine area of the Galicia (NE Spain). Shoot apical parts were selected, EDTA-washed and finally devitalized by oven drying at 100 °C. For the first time, all the variables affecting the air pollutant uptake by moss exposed in bags were considered: 1) the moss bags characteristics (round, spherical and flat shapes; nylon net mesh size of 1, 2 and 4 mm; moss amount and moss weight/bag surface area ratio of 15, 30, 45 mg cm^{-2}); 2) the exposure criteria (exposure time of 3, 6, 12 weeks;

exposure heights of 4, 7, 10 m above ground); 3) the climatic conditions of the exposure area. The concentrations of metals and metalloids were determined by ICP mass spectrometry and the results were evaluated comparing pre- and post-exposure moss samples. Results showed that the amount of moss included in bags was the most important factor affecting the pollutant accumulation by mosses: the more the moss density inside bags increases, the less metal uptake occurs. The other variables (climate, bag size and shape, exposure time and height) had low or no influence at all. As consequence of the obtained results, the project Mossclone proposed the use of a standard moss bag, spherical-shaped, with a mesh size of 2 mm and filled with a moss amount less than 15 mg per cm^2 of bag surface area. In addition, it was suggested to expose the moss-bags for not less than six weeks (to increase the detectability of metal concentrations in moss) and, for practical reasons, at 4 m above the ground.

The proposed standardized protocol for moss-bag exposure was then tested in a biomonitoring campaign carried out in the framework of the LIFE-Ecoremed project, in order to assess the air quality of five municipalities belonging to the Italian RIPS "Litorale Domizio-Agro Aversano" (Campania Region, south Italy). In each municipality, two scenarios (urban and agricultural) and two sub-scenarios (a street side and a corresponding green area) were selected, in order to evaluate the anthropogenic pollution, with a particular attention to the vehicular traffic impact on the surrounding areas. The concentrations of twenty PAHs and of thirty-nine elements including rare earths were determined by ICP mass spectrometry in pre- and post-exposure samples of *Hypnum cupressiforme* Hedw. moss, treated and exposed following the standardized method. After exposure, the concentration of most of the elements and PAHs (in particular the 4- and 5-ringed PAHs) was significantly increased in moss material. The pollutants had a similar spatial distribution pattern over the entire study area, with road traffic and agricultural practices as the major diffuse pollution sources. *Hypnum cupressiforme* moss bags was able to detect airborne element and PAH inputs and to discriminate different pollution levels in a landscape characterized by a jeopardized structure in which agricultural and urban/residential sites are strictly mixed together.

The data obtained in the Italian biomonitoring campaign were combined with those provided by emission inventories, which are a collection of estimations, recorded with spatial disaggregation, on the type, amount and emission sources of pollutants. As a result, it was observed that both approaches (biomonitoring and emission inventory) indicated the same most polluted municipality and a similar spatial pattern, in particular for lead. This suggests that the joint use of emission inventory and moss accumulation could be a valuable resource to reveal contaminants better than the use of a single approach, allowing a more deep investigation on the pollutant emission sources, especially for those contaminants not routinely monitored.

To overcome the limits of the use of native mosses, the last part of the PhD thesis is focused on the characterization of a new moss biomaterial, provided by the cloning inside bioreactors. The cloned moss specie in question is the *Sphagnum palustre* L., whose elemental composition (pre- and post-treatments with EDTA and by devitalization) and molecular profile were given, in comparison with the conspecific field moss, in order to outline a defined fingerprint of the new biomaterial. The morphological and physico-chemical properties of the moss adsorbing surfaces were also examined by electron microscopy, *in vitro* experiments on metal adsorption and by the chemical analysis of the surface exchange sites. A field exposure test with moss-bags was performed, comparing the clone and the naturally grown *P. purum*. The clones exhibited a much lower metal concentration (from 10 to 100 times) in their tissues than the native samples, thus making the former better indicators of low metal loading. New DNA markers, also useful for systematic analyses of the *Sphagnum* genus, were provided in order to characterize and label the clone. The *S. palustre* clone exhibited acid base properties similar to those of naturally grown *Sphagnum* samples and showed a significantly higher metal uptake performance. Therefore, the use of this biomaterial, with very homogenous morphological and chemical characteristics and a remarkable metal uptake capability, is strongly recommended in the view of a rigorously standardized moss-bag protocol for the active monitoring of persistent atmospheric pollutants.

INTRODUCTION

The contamination of the atmosphere and the improvement of air quality still represent a huge and pressing issue for the entire globe. Air pollution involves the whole environment and the human life and leads to a multitude of adverse consequences to the human health, the ecosystems and the climate. Air pollutants deriving from both natural and anthropogenic sources can be transported over long distances and cover large areas in form of wet and dry atmospheric depositions, representing a serious risk factor for human health by inhalation or entrance in the food chain (EEA, 2015). Recently, the World Health Organization (WHO) estimated around 7 million the number of people deaths in 2012 caused by household and outdoor air pollution (WHO, 2014). The EEA recognised particulate matter, sulfur and nitrogen oxides and ground-level ozone as the three pollutants most affecting human health (EEA, 2015). Health effects are related to both short-term and long-term exposure to air pollution. In 2013, the International Agency for Research on Cancer (IARC) defined the outdoor air pollution a human carcinogenic, with the particulate matter (PM) as the most closely associated with increased cancer incidence, especially regarding the lungs. Moreover, PM represents the most important transport form of heavy metal in the atmosphere (WHO, 2013). The toxicity effects of heavy metals for humans are often metal-specific and related to the exposure conditions. Several metals, including the essential elements like iron, copper and zinc, could produce acute or chronic deseases also at low concentrations (EC Report, 2002; Rajaganapathy et al., 2011). The polycyclic aromatic hydrocarbons (PAHs) are widely diffuse in the atmosphere and were one of the first air pollutants defined as a suspected carcinogen. The benzo(a)pyrene is a well-known carcinogenic substance, whose concentrations were estimated being above the risk threshold in several urban areas, especially in central and eastern European areas (Kim et al., 2013).

Based on the concerns above discussed, it is clear that the air pollution appears a very complex and arduous issue to resolve. It poses a challenge in terms of management and mitigation and requires a continue updating of the air quality status, extensive investigation on primary and secondary pollutant sources, a deep understanding of the dispersion mechanisms of contaminants and a good knowledge of their impact on ecosystems and human life. In this direction, the European Union adopted specific directives on the assessment and management of the air quality (the 96/62/EC and the later 2008/50/EC) with the obligation to report periodically the air quality status for each member state and to set strategies to improve air quality and to abate its pollution and consequently the risks for human health.

However, the presence of pollutants in the atmosphere is not easy to monitor, as well as the acquisition of realistic information on their concentrations and emission forms, as the releasing trends of pollutants in the atmosphere vary in space and time. The data on air quality collected by monitoring devices used in compliance with the EU Directives (e.g. passive samplers, continuous analyzers), are too limited in number although often accurate and obtained within relatively short period. Consequently, these data are not adequate to describe spatial-temporal trends of air pollutants. Mostly, the monitoring stations are installed only in urban and peri-urban areas, thus lacking of representativeness of a larger area to which they refer. Mathematical models could help to estimate the pollution status in the areas not covered by monitoring devices, but they are not reliable due to their intrinsic uncertainty. Due to high economic demands and technical limitations associated with the use of monitoring devices and with the analysis of the air contaminants, only a few number of pollutant is monitored (mainly CO, SO_x, N_xO_y and PMs), without paying attention to the equally toxic compounds (i.e. lead, cadmium, arsenic, nickel, mercury and PAHs) included in the abovementioned Directives. As a result, there is a lack of representative data useful on large scale for the management and the assessment of air quality in Europe.

To overcome all these limits, the biomonitoring represents a good solution for air quality monitoring. The biomonitoring is based on the use of organisms, or communities of organisms, to obtain quantitative and qualitative information on the environment status and its possible deviation from a normal-control condition due to the atmospheric pollution. Biomonitor organisms like bryophytes, lichens and algae can be considered bioindicators or bioaccumulators. A bioindicator is sensitive to pollution and allows indirect air quality estimations through its morphological, physiological and genetic changings, evaluable at individual, population or community level, due to exposure to pollutant. By contrast, a bioaccumulator is an organism resistant to pollutants, thus making possible to trace the pollution levels in the atmosphere by evaluating the concentrations of pollutants (e.g. heavy metals, organic compounds and radionuclides) adsorbed or accumulated in their tissues (Cenci, 1999).

Unlike the official monitoring methods, the use of bioaccumulators allows: a) to rapidly provide pollution data and temporal trends with high-density measurement points; b) to individuate areas at real or potential risk of exposure to pollutants, especially in view of epidemiological investigations; c) to evaluate the effectiveness of the measures adopted for reducing pollutant emissions; d) to individuate possible pollution sources; e) to validate models describing the long-range transmission and deposition patterns of the pollutants at different spatial scales. In addition, the biomonitoring is a cost effective and easy-to-manage method that fully complies the European directives on air pollution, ensuring their implementation (e.g. Giordano et al., 2005; Aničić et al., 2009; Ares et al., 2010).

Among bryophytes, terrestrial mosses have been employed for years as bioaccumulators in air quality biomonitoring studies. As clear example, an European moss Biomonitoring Network was established in 1990 to estimate atmospheric heavy metal depositions. Currently, this network is coordinated by the UNECE ICP Vegetation Programme (United Nations Economic Commission for Europe, International Cooperative Programme on Effects on Air Pollution on Natural Vegetation and Crops) and involves more than 30 European member for biomonitoring of the air pollution by the use of native moss species. Through a data collection repeated at five-year intervals, the programme aims to characterise pollutant sources and deposition patterns, to define the trends of long-range transboundary air pollution and to estimate the effectiveness of air pollution abatement policies within Europe (i.e. LRTAP Convention; an overview in: Harmens et al., 2015).

The most employed moss species for biomonitoring purposes belong to the genus *Sphagnum*, followed by *Hypnum cupressiforme* Hedw., *Pseudoscleropodium purum* (Hedw.) Fleisch and less often *Pleurozium schreberi* (Brid.) Mitt. and *Hylocomium splendens* (Hedw.) Schimp. In general, the selection of a particular specie is mainly related to its presence and abundance in the study region.

Compared to the higher plants, mosses possess several advantageous characteristics that make them particularly suitable for air pollution monitoring. The mosses are poikilohydric organisms (i.e., they have a state of hydration controlled by environment). Their root system (i.e. rhizoids) is not involved in mineral nutrition but mostly in substrate anchorage and the leaves do not possess a foliar cuticle or thick cell walls as natural barriers (e.g. the thickness of the phylloid cells organized as monolayer could be lower than 10 μm). Moreover, the configuration of moss tissues and the cell organization assure a high surface/mass ratio and a direct exposure to the external environment and, hence, to pollutants. Consequently, the nutrient supply, as well as the uptake of unessential or toxic substances, derives exclusively from wet (e.g. rainfalls, humidity) and dry (dusts) mineral depositions, and involves the entire gametophyte surface (Brown, 1982). Therefore, it is possible to correlate the chemical composition of mosses to the concentration of trace elements present in the atmosphere (Bargagli, 1998, 2006; Jalkanen et al., 2000; Figueira et al., 2002). Another advantageous characteristic is the substantial cation exchange capacity, variable among species (Büscher et al., 1983), combined with high saturation levels (in particular for heavy metals).

The processes involved for mineral accumulation within the moss tissues are specific for different elements, moss specie and environmental conditions and the efficiency of element retention depends on the number and nature of the extracellular binding sites, tissue age and growth condition (Brown and Bates, 1990). Elements in gaseous and soluble form or retained in mineral particles can be adsorbed and bioaccumulated by exploiting ionic bonds with charged chemical groups of the cell wall constituents (mostly carboxylic acids, polyuronic acids, phosphodiester, phosphoryl, amine and polyphenols), intercellular spaces or uneven surfaces (Craigie and Maass 1966; Brown 1984; Wells and Brown 1990; González and Pokrovsky, 2014).

The mosses employed as biomonitors could detect the presence in the atmosphere of several polluting substances (i.e. metals and metalloids, radioactive isotopes, organic compounds like PCDD/PCDF and PCB). It is worth to note that most of these substances, apart from those regulated by legislation (e.g. ozone, sulphur dioxide, carbon monoxide), are not included in European directive about air quality due to the lack of specific techniques for their detection. The pollutants detected by mosses are not only in ionic and gaseous form, but also present as very heterogeneous and complex particles (particulate matters PMs, mostly PM_{10}) and particle aggregates. A lot of metals and metalloids incorporated or adsorbed on these particles are found entrapped within and onto mosses, by virtue of tissue morphology and structural conformation, resulting very helpful to derive information about moss growth environment (Giordano et al., 2005; Adamo et al., 2008b; Tretiach et al., 2011). Another advantage derived from the use of mosses is the possibility to analyse simultaneously different pollutants using the same sample and to increase by several order of magnitude by bio-concentration the content of pollutants present at trace levels in the air, facilitating, consequently, further analytical determinations.

In the areas where the “passive” biomonitoring (collection of pollution data using autochthonous species) is not possible to execute, especially those characterized by hard or scarce moss growth (e.g. urban and indoor environments, mainly due to ecological or pollution factors), the moss “active” biomonitoring (use of transplants, harvested in pristine areas or far from pollution sources) represents a valid solution. Its application resulted progressively increased during last decades, allowing to rule out the variable presence/absence of the target organisms.

Among the different uses of moss transplants for biomonitoring purposes, the active biomonitoring method based on the use of the *moss-bags* is the most applied. It consists of the exposure in the sites to monitor of mosses included in small, round-shaped envelops, generally in nylon. The transplant of mosses in bags allows the exposure of the material according to a rational scheme; the monitoring campaigns can be easily repeated and it is possible to integrate the collected information over the whole exposure time, independently of transitory changes in pollutants (e.g. Adamo et al., 2003). In addition, the evaluation of the pre-exposure concentrations of each investigated pollutant permits the calculation of enrichment rates and a better interpretation of temporal variability of the concentrations.

The first use of moss bags in air pollution monitoring assays dates back to more than 40 years ago when Goodman and Roberts (1971) used moss transplants for monitoring the air quality in an urban/industrial area in South-West Wales, evaluating the accumulation rates by comparison with background pollutant values. Their method was a little modified by Little and Martin (1974) and then employed for years following protocols undergone to continue and several variations during time (for a review, e.g. Ares et al., 2012).

The great limit associated to the application of the moss-bag technique is the lacking of a standardized protocol for pre-treating mosses and for exposing them in the area to monitor. Until now, numerous and different protocols were applied, following often not validated criteria. Without a well-established procedure, it is not possible to compare data from biomonitoring surveys conducted during years and in different world areas. Consequently, the quality of the experimental studies decreases and the significance of the conclusions reached results very low. In this manner, the biomonitoring remains applicable only for scientific purposes, not useful for providing reliable pollution data of a particular interest site, and thus not recognisable by European legislations as official method for the air quality assessment.

Some steps were done in this sense. In June 2007 the European Committee for Standardization proposed a standard method, but it concerns only the use of mosses for passive biomonitoring (CEN/TC 264/WG 31). Actually, the studies addressing all the methodological aspects concerning active biomonitoring by moss-bags are not enough to realize a standardized protocol. The parameters and variables related to moss-bag approach were rarely considered or not properly examined, and often the moss-bag performance was evaluated taking into account one or few aspects at a time.

The main aspects to investigate for a fully standardized moss bags methodology are: 1) the characteristic of the moss specie to include in bags; 2) the moss pre- and post-exposure treatments; 3) the preparation steps and the characteristics of moss-bags 3) the exposure conditions (the location, height from ground and the exposure duration of the moss bags).

For years, mosses have been used in biomonitoring as simply water-washed living materials, but recently the use of devitalized transplants has been suggested. Different devitalizing procedures have been tested (e.g. acid washing, oven-drying, etc.) and the oven drying devitalization treatment seemed a less destructive and more eco-friendly treatment compared to the acid washing. The devitalization does not affect the moss morphology and nullifies the interference of metabolism on passive uptake of pollutants by surfaces, allowing a more direct correlation between the concentrations of the pollutants adsorbed by mosses and the pollution levels of a site of interest (Tretiach et al., 2007; Giordano et al., 2009; Adamo et al., 2011).

The typology of bag employed is a factor influencing the pollutant uptake by mosses. In the available scientific literature moss-bags with different shapes (i.e. spherical, rectangular or square), sizes and moss weights were described (see as examples: Lodenius, 1998; Fernández et al., 2004; Makholm and Mladenoff, 2005; Zechmeister et al., 2006; Tretiach et al., 2007). Other aspects, in particular the exposure time and the height for positioning the moss-bags, could affect the moss-bags performance. Different exposure times should be selected in relation to the pollution levels (Giordano et al., 2009). For biomonitoring surveys, moss bags have been placed at different heights, from ground level to 28 m (see e.g. Huttunen et al.,1981; Culicov et al., 2005; Samecka-Cymerman and Kempers, 2007; Rivera et al., 2011), and with different exposure periods, from 2 weeks to 20 months, but more often limited to 4-8 weeks (see e.g. Evans and Hutchinson, 1996; Tavares and Vasconcelos, 1996).

In general, all the aspects concerning the moss bags preparation and exposure are not deeper investigated and it is still not clarified how they could affect the moss uptake of pollutants from atmosphere.

Another great limit of the active biomonitoring by moss-bags is due to the use of naturally grown mosses as transplants.

Firstly, the preparation of moss bags strictly depends on the availability of moss material to collect from pristine areas. Lots of moss species, especially those most suited for biomonitoring surveys, are not so easy to find, especially considering that their growth and diffusion could vary in relation to natural and anthropogenic causes. In fact, mosses could hardly grow in sites like the industrial and urban ones characterized by relatively high pollution levels that could cause metabolism alterations or physiological stress in mosses (Zechmeister et al., 2003). Moreover, some laws severely limit moss harvesting, in order to avoid species extinction and the alteration of the biodiversity and of the ecosystem equilibrium.

Further, transplants of naturally grown mosses present an intrinsic variability in terms of chemical composition. The composition in elements (pollutants included), as well as the morphological traits, could change in the same moss specie not only if collected in different sites, but also if harvested in the same area but in different seasons (Fernández, et al., 2002; Couto et al., 2004). As a consequence, there is variability in the initial conditions of moss-bags over time and a high degree of uncertainty in the results, which usually derive by comparing the element concentrations in post- and pre-exposure mosses (the latter considered as “control”). Recent studies showed that the moss pre-treatment consisting in careful and several water-washings, although being helpful in reducing the variability of bio-monitoring results (Tretiach et al., 2007; Adamo et al., 2008a), is not enough to preserve the moss vitality during exposure in bags, neither to reduce the trace element content in the control-mosses. Ultimately, the active biomonitoring should be performed using moss transplants with similar and defined initial conditions (e.g. same initial element content and comparable physiological status; Godinho et al., 2008).

In order to overcome this issue, the Mossclone project proposed the use of devitalized cultivated mosses as a tool for air pollution monitoring. An *in vitro*-cultured moss is an available and a ready-to-use material, more homogeneous in terms of physic-chemical composition. In fact, the cloned moss, compared to species collected in nature, has an elemental content less dependent on the external environment; moreover, its laboratory production nullifies the negative impact of the moss harvesting on the ecosystem equilibria.

The moss specie proposed by Mossclone project as new biomaterial to cultivate *in vitro* for biomonitoring surveys is *Sphagnum palustre* L. The *S. palustre* cloning was performed in bioreactors under highly controlled conditions, following a cultivation protocol established by previous studies on the model organism-moss *Physcomitrella patens* (Hedw.) Bruch & Schimp. (Beike et al., 2015). The selection of the specie *S. palustre* was done on the basis of a well-established knowledge about its use as bio-monitor and of studies on its physic-chemical properties. In particular, González and Pokrovsky (2014) evaluated the metal adsorption capacity of four naturally growing moss species commonly employed for moss bag technique, characterizing also their surface binding sites potentially involved in the adsorption processes of pollutants. The *Sphagnum* sp. was compared with *Hypnum cupressiforme* Hedw., *Brachytecium rutabulum* (Hedw.) Schimp., *Pseudoscleropodium purum* (Hedw.) Fleisch. and it resulted the species with the highest metal uptake capacity.

Actually, some issues are still open regarding active biomonitoring by moss-bags. The aspects concerning the preparation and exposure of the moss bags are not deeper investigated, neither it has been clarified how they could affect the moss uptake of pollutants from atmosphere. Little is known about the relation existing between the concentrations of pollutants accumulated in mosses and those estimated by mathematical models and by other monitoring methods, neither it is clear how to discriminate different pollution levels and inputs in heterogeneous areas or sites characterized by overlapping of different pollution sources. Moreover, the mosses employed as biomonitors, as well as the cloned *Sphagnum palustre* proposed by MOSSCLONE project as new biomaterial, are poorly characterized in terms of chemical composition and ability to uptake pollutants; these aspects represent, in fact, key steps for testing the suitability of mosses as biomonitors of air pollution.

OBJECTIVES

The moss-bags technique, although employed for more than 40 years for the active biomonitoring of air pollution, still lacks of a standardization. As consequence, the pollution data from biomonitoring surveys obtained during years are not directly comparable and the use of mosses as biomonitors cannot be recognizable by the European legislation as valid tool for the assessment and management of the air quality. At this moment, the use of mosses for biomonitoring is limited to scientific purposes.

In this context, part of this thesis aims to provide the best options for the implementation and the testing of a standardized protocol for air pollution biomonitoring surveys by the use of moss-bags.

In the **Chapter 1**, all the criteria adopted for design a standardized protocol are described. For the first time, in the framework of the Mossclone project, all the variables influencing moss uptake in bags were taken into account, considering also the geographical-climatic area chosen for the exposure: the moss bag characteristics (shape, nylon net mesh size, amount of moss and moss weight/ bag surface area ratio), the exposure time and the height above ground for the location of the moss bags.

The **Chapter 2** shows the application of the moss-bags method on a small territorial scale, carried out, according to the standardized procedure described in the Chapter 1. The study area is the RIPS (Regional Interest Priority Site Region) of the Campania Region in southern Italy, investigated in the framework of the LIFE-Ecoremed project. The aim is to test the moss-bag standard methodology for describing the pollution degree of a jeopardized territory characterized by different land uses and by a variety of pollutant inputs, and for discriminating pollution levels in sub-areas relatively close each other.

The data obtained from this biomonitoring campaign conducted in the Italian territory were combined with those provided by the emission sources inventory. The **Chapter 3** describes the merging of information obtained from moss-bags exposure and the estimations provided by collection of data (with spatial disaggregation) on the type, amount and emission sources of pollutants. The aim is to test the moss bag method versus a monitoring system currently adopted by EU legislations, highlighting the importance of integrating the two approaches for a better evaluation of the air quality.

An important aspect to consider is that mosses usually selected for active biomonitoring are naturally growing species. For this reason, they present an intrinsic variability in terms of elemental and chemical composition, a factor that easily leads to misinterpretation of the results. In addition, it is often hard to collect native mosses due to their scarce diffusion or the limits imposed by laws on the protection of the species, making impossible to plan experiments with a high degree of replicability. No less importantly, the mechanisms involved in adsorption of pollutant by moss surfaces are rarely investigated; thus, the uptake ability of mosses is still an aspect not totally understood.

To overcome these limits, the MOSSCLONE project proposed the use of a cloned moss for biomonitoring purposes. On the basis of literature data and comparative studies, *Sphagnum palustre* L. was the moss species selected for cloning. The aim of the final part of this PhD thesis is to deeper investigate the characteristics and the properties of the cloned moss, in order to allow a larger employment of this novel biomaterial. In the **Chapter 4** and **Chapter 5**, a molecular and chemical profile of the cloned moss is provided. An exposure field test is performed to verify the performance of moss bags filled with the clone. Moss surfaces are also studied, both at morphological and physic-chemical level (e.g. physical structure, specific surface area, porosity, surface charge), and the clone uptake capacity is tested as function of different parameters (time, pH, metal concentration in solution).

Chapter 1

Best options for the exposure of traditional and innovative moss bags: a systematic evaluation in three European countries

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Abstract

In order to develop an internationally standardized protocol for the moss bag technique, the research team participating in the FP7 European project "MOSSclone" focused on the optimization of the moss bags exposure in terms of bag characteristics (shape of the bags, mesh size, weight/surface ratio), duration and height of exposure by comparing traditional moss bags to a new concept bag, "Mossphere". In particular, the effects of each variable on the metal uptake from the air were evaluated by a systematic experimental design applied in urban, industrial, agricultural and background areas of three European countries with oceanic, Mediterranean and continental climate. The results evidenced that the shape, the mesh size of the bags and the exposure height (in the tested ranges), did not significantly influence the accumulation capacity of the transplanted moss. The aspects more affecting the element uptake were represented by the density of the moss inside the bags and the relative ratio between its weight and the surface area of the bag. We found that, the lower the density, the higher the uptake recorded. Moreover, three weeks of exposure were not enough to have a consistent accumulation signal in all the environments tested, thus we suggest an exposure period not shorter than 6 weeks, which is appropriate in most situations. The above results were confirmed in all the countries and scenarios tested. The adoption of a shared exposure protocol by the research community is strongly recommended since it is a key aspect to make biomonitoring surveys directly comparable, also in view of its recognition as a monitoring method by the EU legislation.

Capsule

Variables significantly affecting moss bag uptake are exposure time and moss weight/bag surface ratio.

Keywords: *Active biomonitoring*; *air pollution*, *moss uptake; trace elements; Mossphere; Pseudoscleropodium purum.*

Highlights

- Variables affecting moss bag uptake were tested by systematic experimental design.
- Variables investigated were: shape, mesh size, density, height and exposure time.
- A new concept bag "Mossphere" was developed.
- Uptake effectiveness was evaluated in four scenarios of three EU countries.
- The variables most affecting uptake are moss density and exposure time.

1. Introduction

Outdoor air pollutants are a complex mixture of primary and secondary compounds originating from a myriad of natural and anthropogenic sources. Although evidence of specific components of this mixture to drive major risk for human health remained for long inconclusive, the particulate matter (PM) has recently been designed as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC). Fine airborne particles contain metals, polycyclic aromatic hydrocarbons (PAHs) and other toxic chemicals and can increase the natural-cause mortality even at concentrations well below the European annual mean limit value (Beelen et al., 2014). Although some metals in (wet, dry and occult) atmospheric deposition are among potentially toxic pollutants, in Europe air quality target values have been established only for As, Cd, Ni and Pb and most automatic monitoring stations measure the concentrations of particles based on size (aerodynamic diameter of PM_{10}: ≤ 10 µm or $PM_{2.5}$: ≤ 2.5 µm) and not their chemical composition. Moreover, when some data on metal deposition are available they have poor spatial coverage and local sources or variations in their fluxes remain hidden. Thus, to obtain quantitative information on the spatial deposition of metals (especially those not measured by monitoring devices) the monitoring with suitable organisms has become a very common approach (e.g., Harmens et al., 2015 and references therein). The moss bag technique is probably the most applied method for the active monitoring of airborne trace elements in urban and industrial environments. This technique was introduced by Goodman and Roberts (1971), later modified by Little and Martin (1974) and during the last decades there were several investigations pointing to the optimization and standardization of the method (e.g. Gailey and Lloyd, 1986a, b, c, d; Ares et al., 2012; 2014; Giordano et al., 2013). However, most studies considered only one of few methodological steps of the moss bag technique such as the duration or the height of bag exposure and were developed in areas with specific climatic and environmental conditions (e.g. Ares et al., 2012).

Taking advantage of the FP7 European project “MOSSclone” focused on the culture of a particularly performing moss clone, the production of a new concept bag (“Mossphere”) for the moss exposure and the development of a standardized protocol for the moss bag technique, the research team involved in the project undertook a complex and systematic evaluation of the most important variables affecting the results of metal biomonitoring with moss bags. In particular, the effects of each variable (1. shape of bags; 2. net mesh size, 3. ratio between moss weight and bag surface area; 4. duration of the exposure, 5. height of the exposure) on the metal uptake were evaluated separately. In order to develop an internationally standardized protocol for the moss bag technique it seemed necessary to test the variability in each of the methodological steps in a range of climatic conditions and land use classes. To this end an experimental design was, for the first time, applied in urban, industrial, agricultural and background areas of three European countries with oceanic, Mediterranean and continental climate.

2. Material and Methods

2.1 Preparation of moss transplants

Moss transplants were prepared with *Pseudoscleropodium purum* (Hedw.) M. Fleisch., one of the most commonly used species in the moss bag technique (Ares et al., 2012). Samples were collected in a background area of SE Galicia (NW Spain; X: 596060, Y: 4709910 UTM 29N ETRS89) selected on the basis of previous results (Boquete et al., 2013).

In the laboratory 5 cm long green apices were selected. This material was firstly cleaned by placing in a plastic sieve (0.7 cm mesh size) and then subjected to one wash of 20 min with 10 mM EDTA (12.5 g d.w. moss/1 L EDTA with shaking) and three washes of 20 min each with distilled water (10 g d.w./1 L distilled water with shaking) and washed 3 times for 10 min in bidistilled water (10 g d.w./ 1 L bidistilled water with shaking) to remove adhering soil particles. The samples were then blotted on filter paper to remove excess moisture. Afterwards, moss apices were devitalized following three consecutive drying cycles of 8 hours each at 50 ºC, 80 ºC and 100 ºC. Finally, the bags were prepared (see below), vacuum packed and stored until use.

2.2 Experimental set-up

All the field experiments were carried out in NW Spain, SW Italy and E Austria (Fig.1). The climate in Galicia (NW Spain) is influenced by the ocean and is temperate maritime; high rainfall (1000-2000 mm per year) and mild temperatures (annual average, 13 C° and spring average, 15 C°) characterize the investigated area (www.meteogalicia.es). The climate in Campania (SW Italy) is mild and influenced by the sea, (annual average temperature, 10.5 C° and spring average, 13.5 C°). The annual rainfall ranges between 900 e 1200 mm (http://www.sito.regione.campania.it/). Austria is not bordering the sea and presenting a temperate/continental climate in the investigated area (annual rainfall 550 - 900 mm; annual average temperature, 11.3 C° and spring average, 6.5 C°) (http://www.zamg.ac.at/).

In each country seven exposure sites (ESs) affected by different degrees and types of contamination were selected and classified accordingly as agricultural, background, industrial, and urban sites. The bags were hung vertically from sticks of an inert material fixed perpendicularly to a pole, or similar structures, at a height of 4 m above the ground, except in experiment 2.2.5 (see below). The moss bags were exposed for 3 weeks, except in experiment 2.2.4 (see below). Three replicates per ES for every single treatment were exposed. Ten moss bags, vacuum-packed in polyethylene bags and stored at 4 ºC, were used to check contamination after exposure during transportation and laboratory handling.

2.2.1 Shape of the bags

Two couplets of moss-bags of different shape were compared at parity of mesh size (2 mm), quantity of devitalized moss filled in, and external surface of the device: rounded bag vs. Mossphere (S_{30}) – both made with a dry mass/surface ratio of 30 mg/cm^2 - and flat bag vs. Mossphere (S_{15}) – both made with a dry mass/surface ratio of 15 mg/cm^2 -, Figure 2.

The Mossphere is a device designed by our team consisting of two coaxial empty spheres, each formed by two hemispheres, made of pierced high-density polyethylene (the internal sphere), and of a 2 mm mesh nylon net) (the external sphere). The internal sphere is 10 cm in diameter and has 3 mm long spikes homogeneously distributed on the convex side. The external sphere is 11 cm in diameter. The two spheres are closed with four plastic wires passing through four holes in the equatorial plastic border that delimits each hemisphere. The space between the two spheres (10 mm thick) is filled with moss, which is maintained in place by the spikes of the inner sphere.

The rounded bags were made as described by Ares et al. (2014). A square of plastic net of 22 × 22 cm was filled with the moss material, and secured with a nylon thread.

Rectangular flat bags (approximately 700 cm^2) were made with plastic net (2 mm mesh size). The moss was distributed homogeneously inside the bag, and to minimize overlapping and compression of the moss during the exposure (hanging vertically) (Temple et al., 1981), the bag was sewn in a zig-zag pattern with nylon thread to make 3 compartments.

Prior to use, the plastic net was washed in HNO_3 and then in distilled water to eliminate any trace contaminants. Transplants were exposed in triplicate for three weeks in all the ESs in March 2013; total number of samples=189 (3 shapes x 3 countries x 7 ESs x 3 replicates).

2.2.2 Mesh size

Mosspheres with different mesh size (1 mm, 2 mm, and 4 mm) were filled with 11.40 g of dry moss material (dry mass/surface ratio: 30 mg/cm^2) and exposed in triplicate in all the ESs at 4 m above the ground for three weeks in March 2013; total number of samples=189 (3 mesh size x 3 countries x 7 ESs x 3 replicates).

2.2.3 Ratio between moss weight and bag surface area

In order to investigate the effect of weight, Mosspheres with a nylon mesh of 2 mm were filled with 5.70, 11.40 or 17.10 g d. w. of moss material, in order to have weight/surface ratios of 15 mg/cm^2 (W15), 30 mg/cm^2 (W30) and 45 mg/cm^2 (W45), respectively. The Mosspheres were exposed in triplicate in all the ESs at 4 m above the ground for three weeks in March 2013; total number of samples=189 (3 weight/surface ratios x 3 countries x 7 ESs x 3 replicates).

2.2.4 Duration of exposure

For this assay, Mosspheres with a dry mass/surface ratio of 30 mg/cm^2 and 2 mm mesh were exposed in triplicate at 4 m above the ground. Three different durations of exposure (3, 6 and 12 weeks) were tested in parallel in all the ESs between March and June 2013, so there were in total 4 subsequent exposure periods of 3 weeks (n=252; 3 countries x 7 EEs x 4 periods x 3 replicates), 2 subsequent exposure periods of 6 weeks (n=126; 3 countries x 7 EEs x 2 periods x 3 replicates) and 1 exposure period of 12 weeks (n=63; 3 countries x 7 EEs x 3 replicates).

2.2.5 Height of exposure

For this assay, Mosspheres with a dry mass/surface ratio of 30 mg/cm^2 and 2 mm mesh were exposed in triplicate at 4, 7 and 10 m above the ground in 5 ESs of each country, excluding the agricultural and background ones (n=135; 3 countries x 5 ESs x 3 heights x 3 replicates). The experiment was carried out in a single period of three weeks between March and April 2013.

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 heavy metal-free mills (Retsch ZM 200 and Retsch PM100). Moss samples were digested in 1 mL H_2O_2 (30%) and 5 mL aqua regia (1 HNO_3 : 3 HCl) in a microwave (CEM Mars 5) and then filtered. Concentrations of metals and metalloids included in the EU directives (As, Cd, Hg, Ni and Pb), as well as indicators of industrial (Al, Ba, Be, Cr, Co, Cu, Se, Sn, Sr, V and Zn) and traffic (Pd, Pt and Rh) emissions were determined by inductively coupled plasma mass spectrometry (ICP-MS - Varian 820-MS) at TE Labs (Tullow, Ireland). Mercury was determined in an elemental analyzer (Milestone DMA 80). To control the analytical quality, analytical replicates were processed, 1 every 10 samples and the standard deviation of analytical replicates was calculated. Certified reference material (M2 *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 ranged between 88% for Ba to 119% for Ni. The relative standard deviation (RSD) was not higher than 17% (Cr), except for As (50%). The overall error associated with the analytical process was usually lower than 8% and never higher than 19%, with the only exception for As (90%). The concentrations of Be, Co, Pd, Pt, Rh and Sn were under detection limits in the reference material.

2.4 Data analysis

The limit of quantification of the technique (LOQ_T) was calculated from the initial concentrations as follows: $xC_i + 1.96sC_i$, where xC_i is the mean value of the initial concentration in unexposed moss samples (n=10) for each element determined, and sC_i is the corresponding standard deviation (Couto et al., 2004 as modified in Ares et al., 2015). 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. Comparisons between the different exposure options were made separately for each country and were based on those elements showing concentrations higher than the LOQ_T at least in the arbitrarily fixed limit of 60% of the whole dataset. All comparisons were carried out by using a non parametric Wilcoxon matched pairs test (for 2 groups, also used as a post-hoc test when H0 was refused with Friedman ANOVA test) or Friedman (for 3 or more groups) tests by STATISTICA and R software. The ratios between median absolute deviation (MAD) and median were calculated to evaluate the data spread of each solution tested. The selection of the best solution was done on the basis of the effects the various tested options had on the moss uptake and on the replicability of the results.

3. Results and Discussion

3.1 Chemical analysis

As there were no significant 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 moss transportation and handling in the laboratory.

The elements useful for our comparison were Al, Ba, Cr, Cu, Fe, Hg, Ni, Pb, Sr and Zn; all the others were under the detection limit. This outcome was not due to the analytical method applied since we obtained good percentages of recovery (see section 2.3). Probably, in the chosen sites, some elements were present at low concentrations and the exposure period (see section 3.5) was too short. Moreover, the three countries were characterized by different pollution levels, consequently only those elements complying with the criteria explained above were considered (see section 2.4).

3.2 Shape of the bags

According to the arbitrarily fixed limit of 60%, for the comparison between F (flat) and S_{15} (Mossphere) it was possible to use all the elements except Cr and Hg, while for the comparison between R (rounded bag) and S_{30} Mossphere, only Al, Ba, Cu, Ni, Sr (only in Italy) and Zn were useful for our purposes.

3.2.1 Flat vs. S15 Mossphere

Figure 3 shows the results of this assay separately for each country. In Austria, Al and Zn were found mostly accumulated in the moss exposed in the flat bags, while that placed in S_{15} had a higher amount of Ni and Pb. In Italy, a higher signal for Al and Zn was recorded in flat bags, while S_{15} allowed a better accumulation for Ba, Cu, Fe, Ni, Pb and Sr. In Spain, the concentrations of Al, Ba, Cu, Ni, Pb and Zn were higher in the flat bags, no differences in terms of accumulation were observed for the remaining elements. A general trend is clear for the accumulation of Al and Zn, for which the flat bags allowed a higher uptake performance. For the remaining elements, we found uneven outcomes, suggesting that local situations (e.g. weather conditions, orientation of the flat bags) affected the accumulation performance more than the shape *per se*. In terms of data spread, no clear differences were evidenced by the MAD/median ratio (Tab. 1).

Gailey and Lloyd (1986d) reported a better accumulation by the moss *Hypnum* sp. exposed in rounded bags with respect to the flat bags*;* although this is not evident in our results, we support the use of (sub-)spherical bags since they allow a uniform collection efficiency from all space directions. The flat bags in which two dimensions are prevalent on the third one, besides being a handmade product, have the problem that the moss uptake can depend on the exposure orientation. If the pollutants come from a specific direction, the orientation of a flat bag may largely affect the concentrations found in the moss. Anyway, no one of the two tested options seems to be the best in all situations, but both are able to discriminate among the different scenarios (see paragraph 3.7).

3.2.1 Rounded vs. S30 Mossphere

In Austria, the moss exposed in the rounded bags had the highest concentration of Ba, Cu and Zn. In Italy, the rounded bags ensured a better accumulation for Ni and Zn, while Al and Sr where higher in the moss contained in S_{30}. In Spain, the accumulation of Al, Cu, Ni and Zn was higher in the rounded bags, while no differences were found for the remaining elements (Figure 2). There is a clear general trend only for Zn, for which the rounded bag always allowed the highest accumulation. No clear differences in terms of data spread were evidenced by the MAD/median ratio (Table 1). This comparison did not yield a clear result; the two options behaved quite similarly, likely for the same density of the moss material inside the bag (see paragraph 3.4). Probably the higher moss density, joint to the exposure time (3 weeks), resulted in a reduced number of elements useful for comparison.

3.3 Mesh size

Post-exposure values of Ni and Pb never exceeded the respective LOQ_T and therefore were not used in our evaluations; this was true for all the countries (see Figure 3). In Austria, the elements complying with our criterion were Ba, Cu, Fe, Hg and Zn. Only for Ba and Zn the 4 mm mesh seemed to ensure a better accumulation performance, whereas no significant differences were observed between the tested mesh sizes for Cu, Fe and Hg. In Italy, only Al, Ba, Cr, Fe and Sr were above the LOQ_T. The 1 mm mesh appeared to enhance the accumulation of Cr, while the 4 mm mesh was the best option for Ba and Sr. In Spain, the 1 mm mesh was the best option for the detection of Cu and Fe.

Overall, the three mesh sizes differed statistically only for a few elements. In some cases the 4 mm mesh seemed to allow a better performance of the moss material with respect to the other two mesh sizes, but this result was not always confirmed. The few cases in which the 4 mm size appeared to be the best option could be explained by the loss of material (approximately 20 %) from the bags that, as a consequence, caused a reduction of the weight/bag surface ratio and, hence, increased the accumulation (see section 3.4). No clear differences in data spread were found for different mesh sizes (see Table 2); this finding is in agreement with Giordano et al. (2013) who evidenced, for the lichen *Pseudevernia furfuracea* Zopf., the mesh capability to homogenise and reduce the variation in element accumulation and this seemed true independently of the mesh size interval employed. According to different studies, the choice of an inadequate mesh size may lead to the loss of large amounts of material, principally due to weather conditions (e.g. Archibold, 1985; Strachan and Glooschenko, 1988; Ares et al., 2012). We can conclude that mesh size does not affect the accumulation of elements in moss in a significant way; the selection of the proper mesh size must thus take into account only the loss of material during the exposure.

3.4 Ratio between moss weight and bag surface area

It was not possible to carry out the statistical analysis for Cr, as most of the data did not satisfy the 60 % criterion. For all the remaining elements, the final concentrations increased in the Mossphere filled with the smallest amount of moss. The W15 was the only option fulfilling the LOQ_T for most of the elements as the case of: Ba, Fe, Ni and Pb in Austria; Pb and Ni in Italy; Cu, Ni and Sr in Spain. In all the other circumstances, when W30 and W45 also fulfilled the criterion, W15 significantly differed from the other two setups giving always the “largest signal” (Figure 5). In replicability terms W15 showed only in few cases a higher data spread if compared with W30 and W45 (Table 3). Our results reflect in part those of Zechmeister et al. (2006) and Ares et al. (2014): the maximum element interception is gained when the moss material is exposed to the air in a thin layer with all the shoots equally exposed, with no or scarce overlap among gametophores and without leaflets flattening. In particular, Ares et al. (2014), by using *Sphagnum denticulatum* Brid., observed a general increase in the moss post-exposure concentrations of Cd, Pb and Zn when they decreased the moss amount inside the bags.

Moreover the smallest ratio we applied (15 mg/cm^2), did not affect the data spread in a significant way, since it was already adequate to minimize replicability problems. Indeed, this ratio is 3 times higher than that suggested by Ares et al. (2014) as a compromise between pollution signal and data replicability. Therefore, we conclude that the quantity of moss contained within the Mossphere significantly affects the accumulation performance of the device; in particular, the smaller the amount of moss, the better accumulation performance, with a negligible effect on the data spread, particularly when the ratio falls in the interval 5-15 mg/cm^2. This is probably a result of a more homogenized distribution of elements on the surface of the receptor (moss), thereby minimizing analytical variation or loss of elements in exposed material.

3.5. Exposure time

In this experiment all the studied elements showed values above the LOQ_T at least for some countries and exposure times (Figure 6). Aluminium, Ba and Pb were the only elements for which no differences were observed in any country or treatment. In Austria the highest accumulation rates (µg g^{-1} per week) corresponded to 3 weeks for Cu and 12 weeks for Hg. In Italy Cu, Fe, Ni and Sr showed the highest rates after 6 weeks, and Cr and Hg after 12 weeks. In Spain the 3-week exposure period caused the highest accumulation rates for Ni and Sr but in the case of Fe and Zn it was obtained after 12 weeks. Overall, exposure periods of 6 or 12 weeks seem, without great differences between them, to enhance accumulation rates for a higher number of elements. Nevertheless, the 12 week period was the only one showing the highest accumulation rates for at least one element in all the countries. As in the previous experiments, no clear differences in terms of data spread were evidenced by the MAD/median ratio (Table 4).

In the moss bag technique the duration of the exposure period has been one of the most studied aspects (e.g. Galey and Lloyd, 1986c; Tavares and Vasconcelos, 1996; Adamo et al., 2003; Basile et al., 2008, Aničić et al., 2008; Giordano et al., 2009; Ares et al., 2014). In general, the evaluation of the time effect was based on the accumulated concentration of pollutants in the moss and its associated variability; but in a thoroughly review of the literature, in which at least three different exposure times were tested (Ratcliffe, 1975; Galey and Lloyd, 1986c; Tavares and Vasconcelos, 1996; Vasconcelos and Tavares, 1998; Basile et al., 2008, 2009; Aničić et al., 2009), Ares et al. (2012) suggest to assess the effect of this variable in terms of uptake rates. These authors stated that the accumulation rate rarely depend on time. Similar results have been recently reported by Ares et al. (2014) after testing the effect of 4-, 8- and 12 week exposure periods in the accumulation rates of Cd, Cu, Hg, Pb and Zn in *Sphagnum denticulatum* bags exposed to different levels of pollution in Galicia (NW Spain). These authors pointed out that the uptake rate tended to be temporally stable, independently of the duration of the exposure period.

Our results agree with this trend: Al, Ba and Pb did not show differences in uptake rates in any country, whereas in the case of Cr, Hg and Zn only one or two exceptions (out of a total of 10 cases) were identified for the 12-weeks period (i.e. Cr in Italy, Hg in Austria and Italy, and Zn in Spain). All the other elements showed variable results, e.g. higher uptake rates of Cu for the 3 weeks period in Austria compared to 12 weeks in Italy and the same goes for Sr after 6 weeks in Italy and Spain. However, in general terms our results do not show a relationship between exposure periods and uptake rates. Hence, it can be concluded that the best option is to expose the moss bags during 12 weeks because it ensures higher accumulation of pollutants. Variations in metal uptake are likely a result of the deposition mode (dry, wet or occult) and therefore, the longer the exposure, the more homogenized these variations are. Nevertheless, when mosses are exposed in the surroundings of pollution sources, periods of 6 weeks could be enough.

3.6 Effect of the exposure height

It was not possible to carry out the statistical analysis for Cr and Hg, as most of the data did not satisfy the 60 % criterion (Fig. 7). The 7 m height was the only option fulfilling LOQ_T for Al, and Pb in Spain, and the 4 m for Pb in Austria. For Ba, Cu, Ni and Sr it was possible to test the hypothesis in Italy and Spain, without finding supporting results; the same occurred for Fe in Spain. The only element which reached LOQ_T in all cases was Zn; for this element no differences were found in Austria, while significant differences were found in Italy (10 m height) and in Spain (4 m height), being always higher at lower heights. No clear differences in terms of data spread were evidenced by the MAD/median ratio (Table 5).

The vertical profile of contamination is the result of 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, specific location of the source of emission (i.e. the vehicle exhaust pipes - fumes - and wear and tear of wheels and asphalt – particles and the emissions from domestic fuel) or topography of the area (i.e. presence of walls, buildings) (Aničić et al., 2009; Adamo et al., 2011; Vuković et al., 2013). As a result of these processes some authors have found vertical profiles in the transplant concentrations. Adamo et al. (2011) found that *Hypnum cupressiforme* bags exposed at a height of 4 m in a street canyon in Naples were more efficient at retaining contaminants associated to traffic and suspended dust (Al, As, Ba, Co, Fe, Pb, Ti, V, and Zn) than samples exposed at a height of 20 m, which captured contaminants associated with long distance transport and cations of marine origin (i.e. Cr, K, Mg, and Mn). The same was found by Vuković et al. (2013) in Belgrade using *Sphagnum girgensohnii* bags with higher concentrations of Al, Ba, Co, Cr, Cu, Ni, Pb, Sr, V and Zn at lower exposure height (i.e. 4 m) than at higher heights (i.e. 8 and 12 m). These results are consistent to those obtained for Zn in Italy and Spain and for Pb in Austria and Spain in this study. Both elements are related to traffic emissions and abrasion processes (Laschober et al., 2004, Zechmeister et al., 2005, Napier et al., 2008; Thorp and Harrison, 2008).

However, as for the other elements determined in the present work, most authors did not find any vertical patterns of moss bags concentrations in previous studies. Rivera et al. (2011) did not find any differences between Al, As, Cd, Cr, Cu, Mo, Pb, Sb, Sn and Zb concentrations in *Hylocomium splendens* bags exposed on balconies at heights of 3-21 m in Girona (NE Spain). De Nicola et al.

(2013) concluded that there were no differences in Cd, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Pb and Zn concentrations in *Hypnum cupressiforme* transplants among samples exposed at heights of 3, 6 and 9 m in street canyons in Naples. In the same way, Vuković et al. (2013) did not find differences for Ca, Cd, Fe, K, Mg, Mn and Na concentrations in *Sphagnum girgenshonii* transplants exposed at 4, 8 and 16 m in Belgrade (Serbia). Finally, Ares et al. (2014) did not find differences in concentrations of Cd, Cu, Hg, Pb and Zn in *Sphagnum denticulatum* moss bags exposed at 0.5, 1, 1.5, 2, 2.5, 3, 4 and 5 m height in Galicia (NW Spain); although in several instances highest concentrations of all elements were yielded at 5 m height, with the exception of a busy roadside, where the highest concentrations were found at a height of 0.5 m.

According to Ares et al. (2014) the results are highly variable and different contamination processes may be captured at different heights; nevertheless, a specific height must be established to standardize this aspect of the moss bags technique. Taking into account practical considerations (e.g. assessment of the quantities of contaminants inhaled by people from the air and avoiding vandalism), an exposure height of 4 m is recommended.

3.7 Evaluations of different scenarios (urban, industrial, agricultural, background)

Element contents in the post-exposed mosses, regardless of shape and mesh-size, indicated the industrial as the most impacted sites followed by the urban and agricultural, down to the background sites. No one of the above options provided an unambiguous signal, so we conclude that shape and mesh did not have an important influence on the accumulation performance (see sections 3.2 and 3.3) in each scenario. However, this was not true for the weight/ surface ratio, which should be always below or equal to 15 mg/cm^2 to allow a better uptake, and hence a clear discrimination among exposure sites (section 3.4).

It is worth noting that in the industrial scenarios a lower number of differences among the tested times was recorded. This likely depends on the higher pollution level characterizing these sites, thereby allowing a higher uptake rate also after shorter exposure periods. Whereas in the background areas, where lower pollution levels occur, the longest exposure period was always needed to achieve the accumulation in the moss.

The duration of exposure indicated that the 12 week option assured the higher uptake of Cr, Hg and Zn in all scenarios. The same solution was also the best option for Fe and Ni in urban, Ni in agricultural, Al in industrial, Ba and Pb in the background sites. By the way, in this latter scenario, 6 and 12 week exposure allowed a similar enrichment of Al, Fe and Ni. No differences among the accumulation rates of Cu and Sr occurred among the three tested periods, and this was true in all scenarios.

Exposure lasting 6 weeks seems long enough to detect atmospheric trace element occurrence in moderately to highly polluted areas. A longer exposure time might be necessary in clean areas, or for those elements whose concentration in the air is typically low. It should also be taken into account that an exposure longer than 6 weeks, can result in enhanced accumulation of a restricted set of elements, but could also increase the probability of a loss of other elements due to leaching and adverse meteorological conditions (e.g. washing out by rain), even at parity of exposure time, as already evidenced in *Hypnum cupressiforme* and *Pseudevernia furfuracea* (Giordano et al., 2009).

As reported in paragraph 2.2.5 the test on exposure at different heights took place only in the urban and industrial sites. In both scenarios Cr, Fe, Hg and Ni never met the 60 % criterion we adopted, as well as Al in urban sites. In the case of Zn only the 4 meter option reached the threshold. For the remaining comparisons, no differences in terms of accumulation were found among the mosses exposed at different heights. These outcomes further confirm the absence of any vertical gradient of the elemental concentrations in mosses hanged between 4 and 10 m above the ground in the study at hand.

4. Conclusions

The optimization for the exposure conditions, aim of the present study, took into account some aspects already tested by other authors and reviewed by Ares et al. (2012, 2104); the uniqueness of this work is represented by the systematic experimental design applied, which allowed to test separately the variables possibly affecting the moss uptake when the material is exposed in bags. Moreover, the exposure was carried out in three European countries differing in climate, meteorology, and in sites belonging to diverse land use classes, in order to test the uptake effectiveness in different environments.

The results evidenced that the shape and the mesh size of the bags, as well as the exposure height are variables not influencing the accumulation capacity of the enclosed moss. The aspects that affect more the element uptake are represented by the density of the moss inside the bags and the relative ratio between its weight and the surface area of the bag. In fact, in our test we confirmed that the lower the density, the higher the uptake recorded. Moreover, three weeks of exposure were not enough to have a consistent accumulation signal, while only a small difference does occur between 6 and 12 weeks of exposure. The above results hold true in all exposure sites, regardless the different climatic conditions and land use classes. Hence, comparisons among different biomonitoring surveys should be made only considering the data obtained with the same “experimental settings” of those variables most affecting the moss uptake (i.e., species, weight/surface ratio and exposure duration).

According to the reported outcomes, the use of a Mossphere, that is reusable, not “home-made” and with a regular and fixed shape is preferable; it should be prepared with a 2 mm mesh net and a moss content allowing a weight/surface ratio ranging between 5 up to 15 mg cm^{-2} and it should be exposed at 4 meters above the ground (this for practical reasons) for a period not shorter than 6 weeks.

To further reduce variability in the samples and to apply sustainability standards, the use of cloned moss grown *in vitro* (Beike et al., 2015; González et al., 2016a) is recommended, as this material is even suitable to accumulate polycyclic aromatic hydrocarbons (PAHs) (Concha-Grana et al., 2015). Furthermore, the moss material should be devitalized like in our current study, because González et al. (2016b) demonstrated the metabolic activity of living moss material for copper recently.

We support the adoption of a shared exposure protocol by the research community, considering it the key aspect to make biomonitoring surveys directly comparable, also in view of its recognition as a monitoring method by the EU legislation.

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Sitography

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Tables

Table 1 – Mean values of the ratio between median absolute deviation (MAD) and median obtained by using "moss-bags" of different shape (flat= F *vs* S_{15} mossphere; rounded=R *vs* S_{30} mossphere) exposed in Austria, Italy and Spain. Values are expressed as percentage.

Country	Shape	Al	Ba	Cr	Cu	Fe	Hg	Ni	Pb	Sr	Zn
Austria	**F**	13	7	5	7	8	3	17	5	2	13
	S_{15}	11	4	23	12	9	2	20	16	2	28
	R	6	1	16	12	8	2	23	8	3	13
	S_{30}	6	4	4	5	4	3	19	1	1	12
Italy	**F**	8	4	16	3	6	3	19	7	3	9
	S_{15}	3	1	12	7	5	2	9	8	1	17
	R	5	3	29	5	4	2	15	8	3	8
	S_{30}	11	2	13	3	2	4	16	2	2	12
Spain	**F**	14	4	27	8	7	3	14	14	2	12
	S_{15}	5	6	17	3	5	3	15	3	2	20
	R	11	5	7	6	4	3	21	11	2	8
	S_{30}	1	4	8	4	4	3	10	5	3	22

Table 2 – Mean values of the ratio between median absolute deviation (MAD) and median obtained by using Mosspheres with different mesh sizes (1, 2, 4 mm) exposed in Austria, Italy and Spain. Values are expressed as percentage.

Country	Mesh Size (mm)	Al	Ba	Cr	Cu	Fe	Hg	Ni	Pb	Sr	Zn
Austria	**1**	3	4	9	8	6	6	11	5	3	13
	2	6	4	4	5	4	3	19	1	1	12
	4	5	3	17	7	6	4	18	3	3	17
Italy	**1**	6	2	21	4	5	5	11	6	3	6
	2	7	2	24	5	2	2	15	5	2	16
	4	7	2	24	5	2	2	15	5	2	16
Spain	**1**	8	8	13	10	12	2	24	2	4	13
	2	1	4	8	4	4	3	10	5	3	22
	4	6	2	5	6	5	3	13	1	3	15

Table 3 – Mean values of the ratio between median absolute deviation (MAD) and median obtained by using Mosspheres with different moss weight/sphere surface ratio (15, 30 and 45 mg/cm^2) exposed in Austria, Italy and Spain. Values are expressed as percentage.

Country	Weight	Al	Ba	Cr	Cu	Fe	Hg	Ni	Pb	Sr	Zn
Austria	**W15**	11	4	23	12	9	2	20	12	2	28
	W30	6	4	4	5	4	3	19	2	1	12
	W45	2	2	13	9	12	1	16	6	3	14
Italy	**W15**	3	1	12	7	5	2	9	8	1	17
	W30	11	2	13	3	2	4	16	2	2	12
	W45	3	0	11	6	4	1	18	14	2	14
Spain	**W15**	5	6	17	3	5	3	15	3	2	20
	W30	1	4	8	4	4	3	10	5	3	22
	W45	5	1	12	5	5	2	12	4	2	12

Table 4 – Mean values of the ratio between median absolute deviation (MAD) and median obtained by using Mosspheres exposed for 3, 6 and 12 weeks in Austria, Italy and Spain. Values are expressed as percentage.

	Time (weeks)	Al	Ba	Cr	Cu	Fe	Pb	Hg	Ni	Sr	Zn
Austria	**3**	6	4	8	8	6	7	3	20	4	17
	6	8	3	17	4	9	5	2	17	5	13
	12	9	8	13	7	9	9	2	15	3	12
Italy	**3**	6	3	20	3	6	4	2	17	2	10
	6	5	2	24	8	5	3	1	30	2	9
	12	6	3	28	3	4	7	2	11	4	12
Spain	**3**	5	3	17	6	6	9	2	11	2	15
	6	8	5	16	4	6	15	2	21	2	12
	12	7	11	22	6	6	4	4	14	2	18

Table 5 – Mean values of the ratio between median absolute deviation (MAD) and median obtained by using Mosspheres exposed at different heights (4, 7 and 10 m) in Austria, Italy and Spain. Values are expressed as percentage.

	Height (m)	Al	Ba	Cr	Cu	Fe	Pb	Hg	Ni	Sr	Zn
Austria	**4**	2	3	4	6	6	3	1	15	5	23
	7	12	5	19	7	3	3	2	7	6	6
	10	2	3	15	8	4	0	1	11	5	10
Italy	**4**	3	2	6	3	2	0	1	10	1	9
	7	1	3	6	4	6	0	2	23	3	26
	10	5	3	5	4	2	0	4	14	0	16
Spain	**4**	5	6	13	2	4	5	2	10	3	12
	7	12	0	8	4	4	5	2	17	2	8
	10	4	0	10	3	3	0	1	13	1	6

Figures

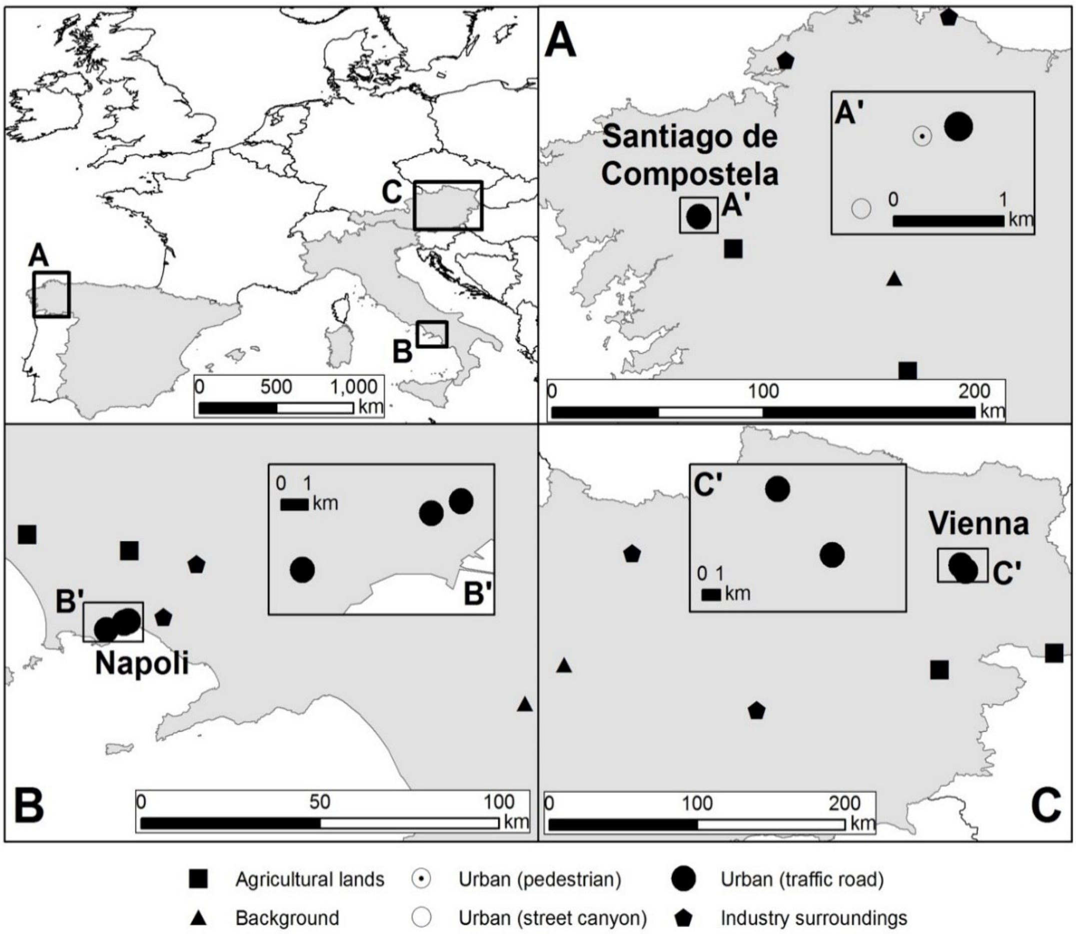

Figure 1. Maps showing the seven stations distributed in industrial, urban, agricultural and background scenarios in Spain (A), Italy (B) and Austria (C).

Figure 2. Different shapes of moss bags tested. a) Rounded; b) Flat; c) Mossphere.

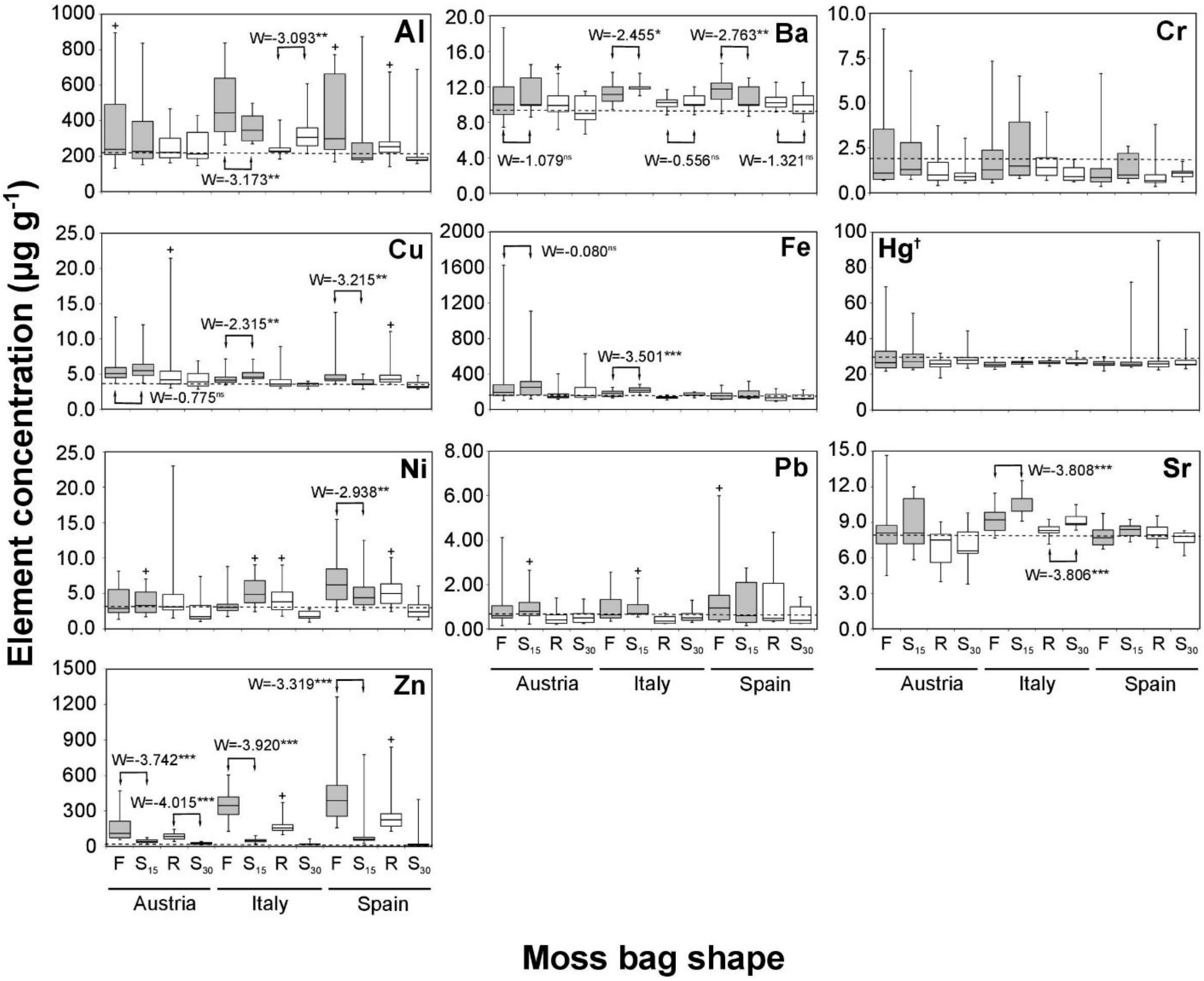

Figure 3 - Box-plots of element concentrations (mg/kg) in the moss exposed in bags of different shape in Austria, Italy and Spain (flat= F *vs* S_{15} Mossphere, grey; rounded=R *vs* S_{30} Mossphere, white). The dashed line represents the LOQ_T. BOX: inside band= median; extremities=1st and 3rd quartiles; whiskers= MIN and MAX. W is the value of Wilcoxon test. "+" indicates the only option(s) fulfilling our criterion.

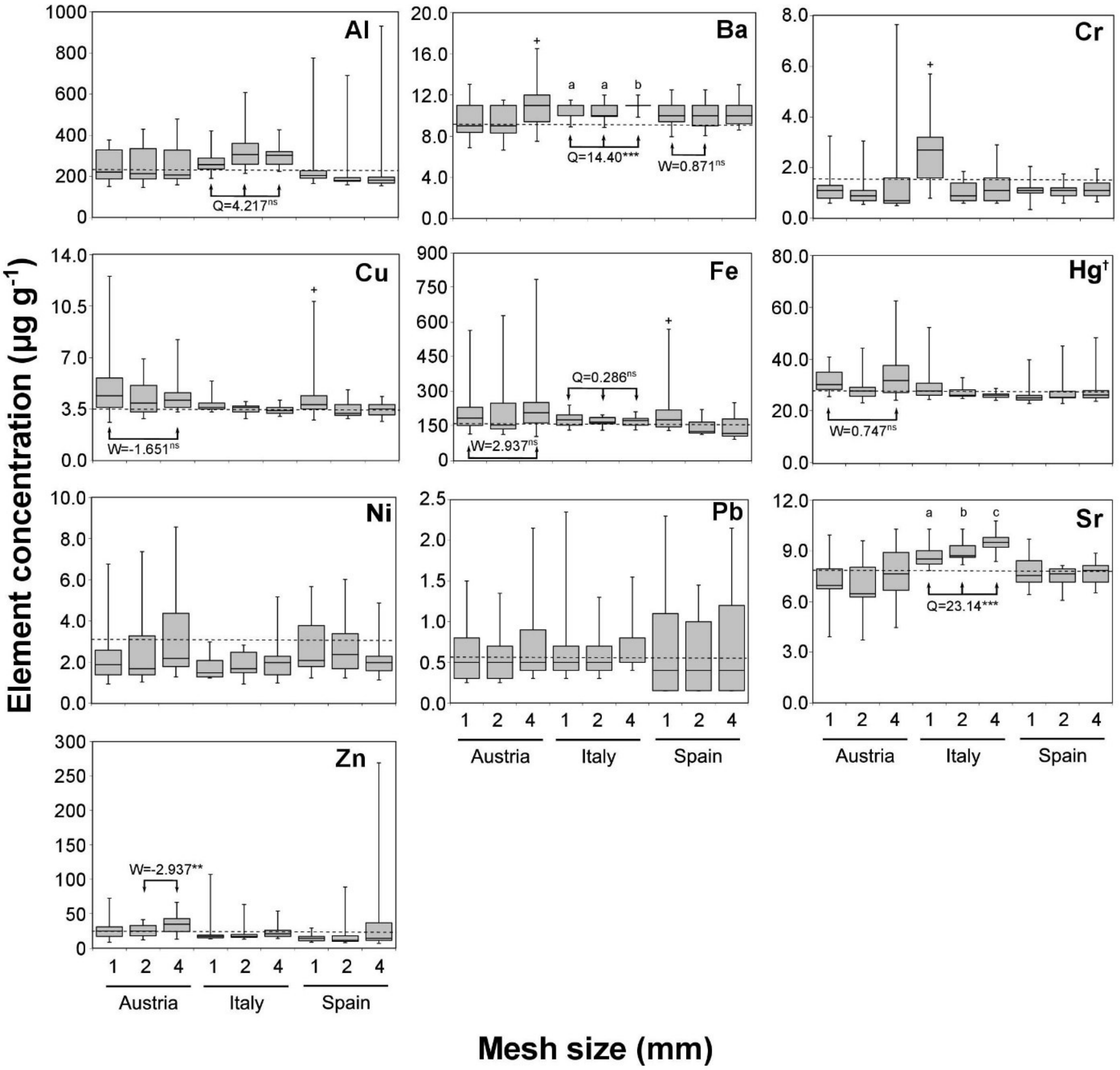

Figure 4 - Box-plots of element concentrations (mg/kg) in the moss exposed in Mosspheres with different mesh sizes (1, 2, 4 mm) in Austria, Italy and Spain. The grey dashed line represents the LOQ_T. BOX: inside band= median; extremities=1st and 3rd quartiles; whiskers= MIN and MAX. W, Q are the values of Wilcoxon and Friedman ANOVA tests. “+” indicates the only option(s) fulfilling our criterion.

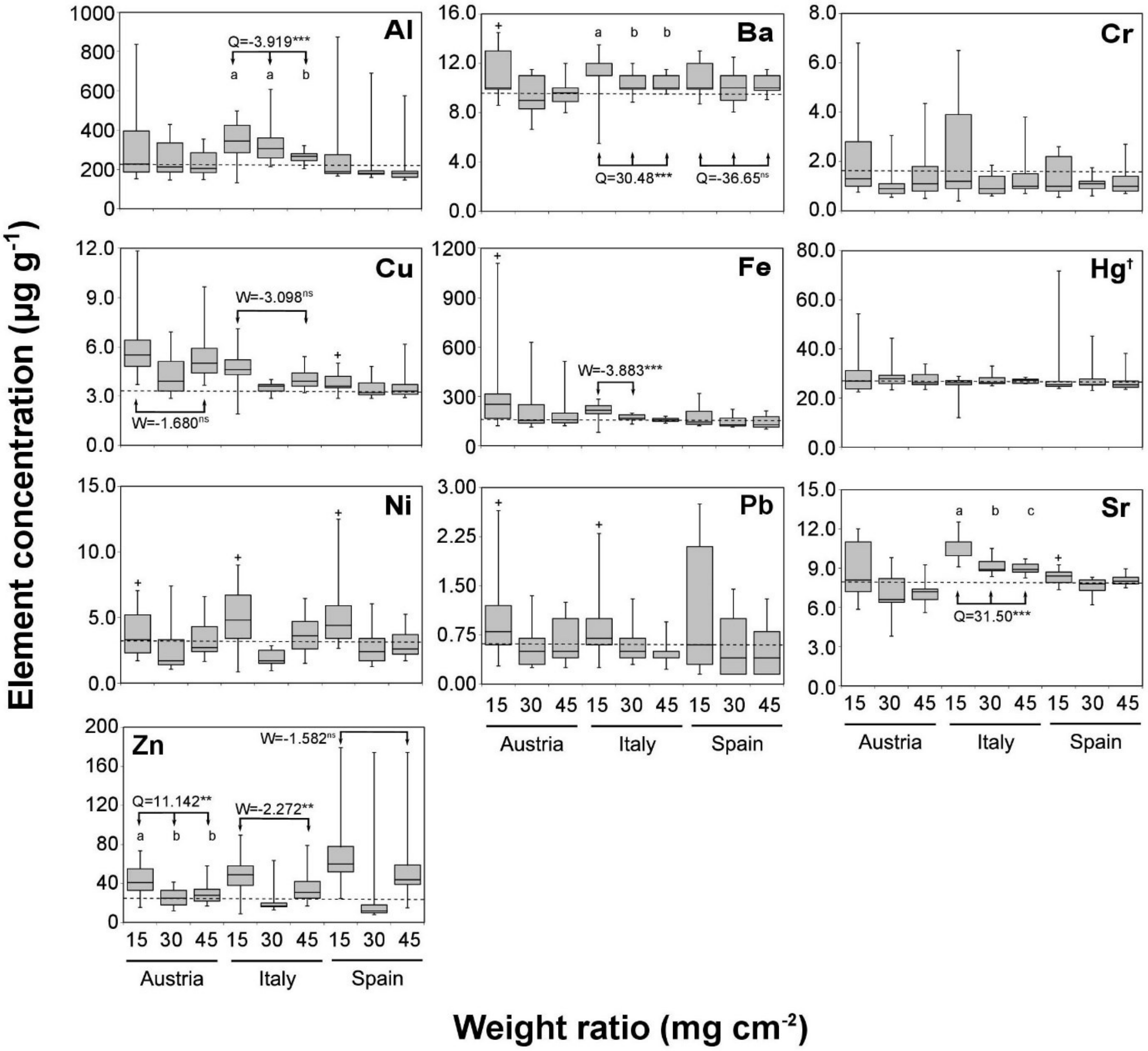

Figure 5 - Box-plots of the element concentrations (mg/kg) in the moss exposed in Mosspheres prepared with different moss weight/sphere surface ratio (W15, W30 and W45) in Austria, Italy and Spain, see text for specifications. The dashed line represents the LOQ$_T$. BOX: inside band= median; extremities=1st and 3rd quartiles; whiskers= MIN and MAX. W, Q are the values of Wilcoxon and Friedman ANOVA tests. "+" indicates the only option(s) fulfilling our criterion.

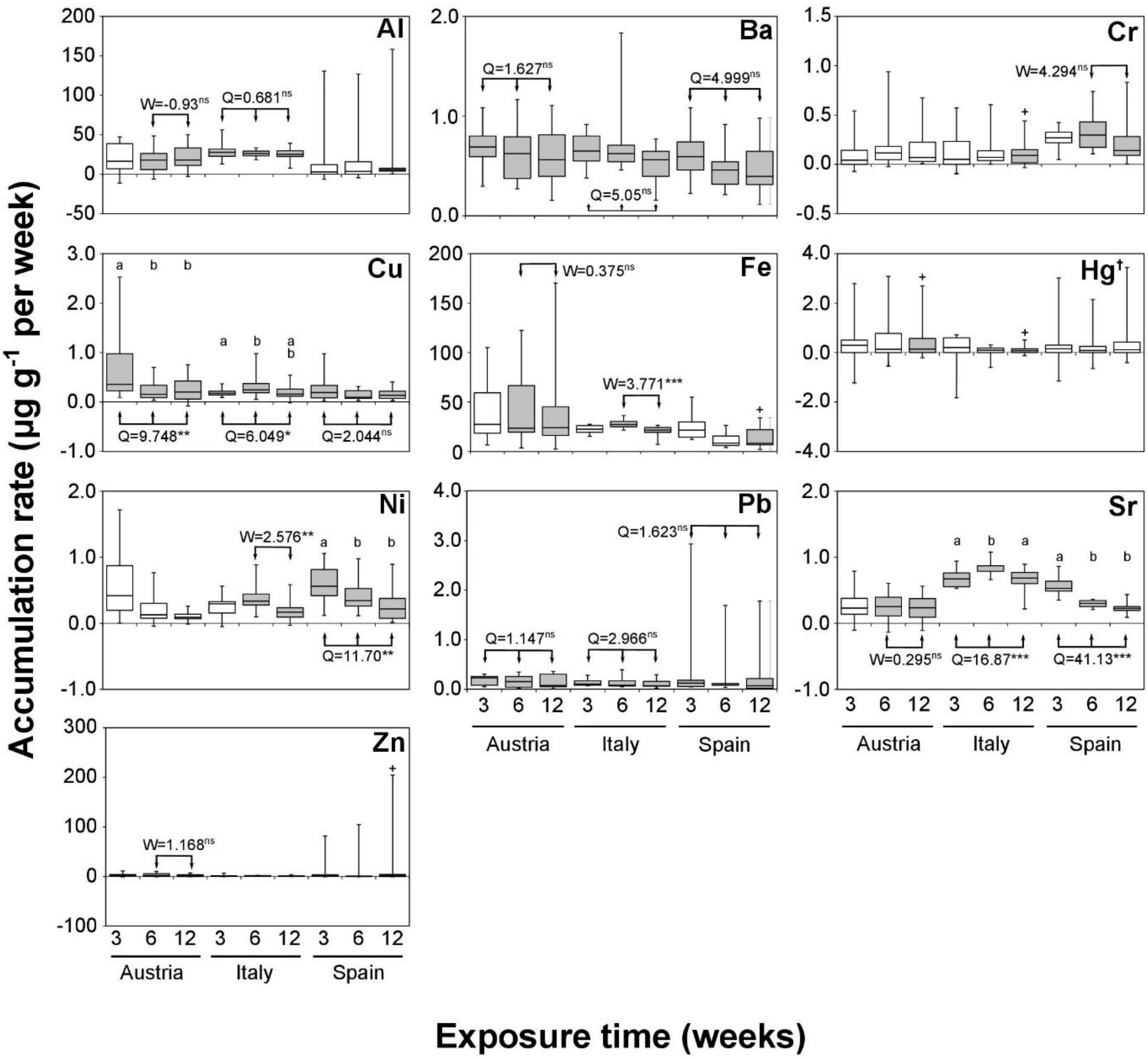

Figure 6 - Box-plots of the element concentrations (mg/kg) in the moss exposed in Mosspheres for 3, 6 and 12 weeks in Austria, Italy and Spain, see text for specifications. The grey dashed line represents the LOQ$_T$. BOX: inside band= median; extremities=1st and 3rd quartiles; whiskers= MIN and MAX. W, Q are the values of Wilcoxon and Friedman ANOVA tests. White boxes: values below the reference criterion; grey boxes: values above the criterion; "+" indicates the only option(s) fulfilling the criterion.

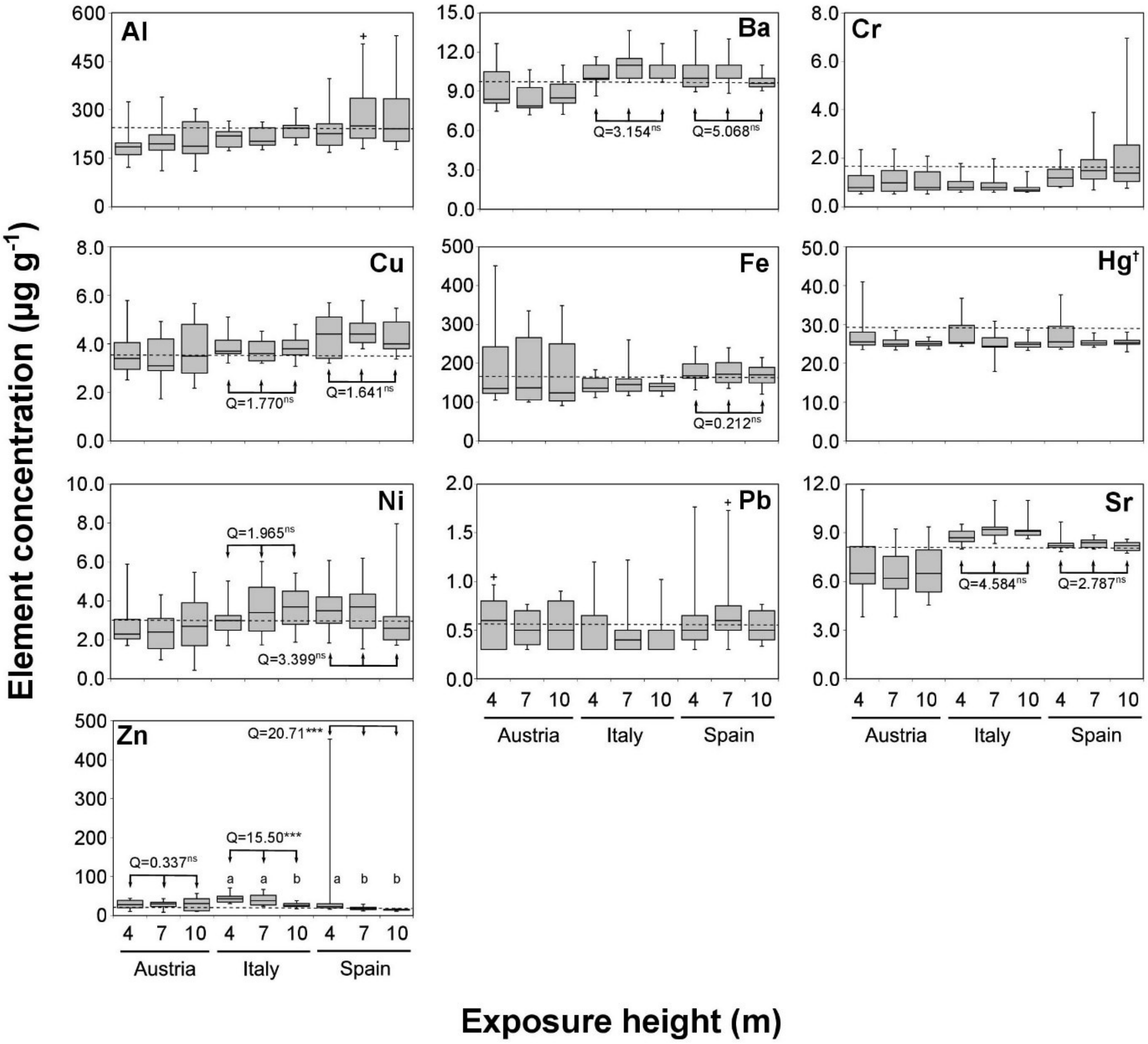

Figure 7 - Box-plots of element concentrations (mg/kg) in the moss exposed in Mosspheres at 4, 7 and 10 m above the ground in Austria, Italy and Spain. The grey dashed line represents the LOQT. BOX: inside band= median; extremities=1st and 3rd quartiles; whiskers= MIN and MAX. Q are the values of Friedman ANOVA test. "+" indicates the only option(s) fulfilling our criterion.

Chapter 2

Biomonitoring of atmospheric pollution by moss bags: discriminating urban-rural structure in a fragmented landscape

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Abstract

In this paper we investigated the possibility to use moss bags to detect pollution inputs - metals, metalloids and polycyclic aromatic hydrocarbons (PAHs) - in sites chosen for their different land use (agricultural, urban/residential scenarios) and proximity to roads (sub-scenarios), in a fragmented conurbation of Campania (southern Italy). We focused on thirty-nine elements including rare earths. For most of them, moss uptake was higher in agricultural than in urban scenarios and in front road sites. Twenty PAHs were analyzed in a subset of agricultural sites; 4 and 5-ringed PAHs were the most abundant, particularly chrysene, fluoranthene and pyrene. Overall results indicated that investigated pollutants have a similar spatial distribution pattern over the entire study area, with road traffic and agricultural practices as the major diffuse pollution sources. Moss bags proved a very sensitive tool, able to discriminate between different land use scenarios and proximity to roads in a mixed rural-urban landscape.

Keywords *Moss transplants; Hypnum cupressiforme; heavy metals; PAHs; atmospheric deposition; urban sprawl.*

Highlights

- Moss bag sensitivity was tested in an urban-rural fragmented landscape.
- Moss uptakes were overall higher in agricultural than in urban sites.
- Mosses were able to discriminate between front road and matching green sites.
- Traffic and intensive agriculture are the major pollution sources in the study area.

1. Introduction

Campania (southern Italy, one of the 20 administrative districts of Italy) experienced in the last century profound changes in land use, as a massive urbanization and increase of industrial settlements, especially in the surroundings of Naples city; it was recently reported that, if from the 1860 to the 1960 the urban/rural structure did not change substantially, in the last 50 years the urbanized area has multiplied its extension by a factor of five. At present, the urban area used by people for residential and productive activities covers approximately 40 % of the entire Naples district, and the area devoted to agriculture the remaining 60 %, producing a complex and jeopardized land (di Gennaro, 2014). This area, historically recognized as one of the most fertile Italian agricultural landscapes, in the last about 10 years was under the attention of media due to a supposed diffuse soil pollution by illegal waste dumping. A part of public and scientific opinion claims that this situation is the cause of increase of some cancers rates and shorter lifespan of people living there (Triassi et al., 2015), while, according to others, and based on the yearly "photograph" of health in Italy made by the Italian Institute of Statistics, Campania stats are progressively approaching those of industrialized northern Italian regions and Europe (ISTAT BES, 2014). Authoritative researchers have recently suggested that Campania could in fact be a perfect field study for a biomonitoring research program, as their poisoned fields could serve as a giant experiment in the new science of 'exposomics', but this suspected link needs to be investigated with appropriate methodologies (Nature editorials, 2014). Air composition and pollution are indeed affected by this complex patchwork of land use, degree of urbanization and spread of productive activities, with the problems related to waste cycle and vehicular traffic fluxes. European Union recognizes the poor air quality as the leading environmental cause of premature death in the EU. This aspect promoted at European level an extensive legislation to reduce harmful pollutants concentrations in ambient air that EU States, including Italy, are trying to fulfill in the last years. Presently, on the basis of European Air Quality Directives (2004/107/EC and 2008/50/EC), besides gaseous pollutants (e.g. NO_x, CO, O_3, SO_2) and particulate matter (PM), only other few atmospheric contaminants (e.g. Pb, Cd, As, Ni, Hg and benzo[a]pyrene) are continuously monitored. For example, the air quality monitoring network in

Campania consists of 27 stations (plus other 9 located near the waste treatment plants) measuring a limited set of pollutants (e.g. NO_2, CO, PM_{10}, $PM_{2.5}$, O_3, SO_2, C_6H_6) (www.arpacampania.it).

The biomonitoring approach was widely used so far to evaluate the environmental quality and detect the presence in the air of inorganic and organic pollutants not routinely measured by conventional monitoring. This methodology is cost effective compared to physic-chemical approach, and can be applied with a flexible experimental design and a higher number of sampling points counterbalancing the lower precision of each single measurement. Particularly, in the last 40 years, mosses transplanted in bags, the so called "moss bags", were used to obtain indications of the inorganic and organic pollutant depositions, especially those linked to particulate matter (for a review see Ares et al., 2012). Surface interception and entrapment of airborne particulate matter is considered the principal pollutant accumulation pathway in mosses, favored by the high surface to mass ratio and cell wall characteristics (e.g. Tretiach et al., 2011).

In this paper, through a specific exposure design, we investigated the capability of moss bags to distinguish pollution inputs in sites with different land use (agricultural, urban/residential) and proximity to roads, in a fragmented landscape of Campania, where the two scenarios are strictly mixed together. An ample set of airborne pollutants (metals, metalloids and PAHs) was analyzed in moss exposed in 40 sites of five municipalities, all comprised in the "Litorale Domizio-Agro Aversano", recognized by the Italian State as a Regional Interest Priority Site (RIPS).

2. Materials and Methods

2.1 The study area

The study area, comprised in the "Litorale Domizio-Agro Aversano", is characterized by a Mediterranean climate and covers five municipalities: Acerra (26 m.a.s.l., 54.71 km^2), Casal di Principe (68 m.a.s.l., 23.49 km^2), Giugliano in Campania (97 m.a.s.l., 94.62 km^2), Maddaloni (63 m.a.s.l., 36,67 km^2) and Teverola (25 m.a.s.l., 6.7 km^2); further details are described in Iodice et al. (2016) and Monaco et al. (2015). In 2004, the territory embracing the towns of Acerra, Nola and Marigliano was named the "triangle of death" by the medical magazine Lancet (Senior and Mazza, 2004) due to the claimed high incidence of cancer-related deaths. More recently, it is better known by the media as "land of fire", in reference to the numerous waste burnings (Legambiente Report, 2003). Despite this perception encouraged by the media, the area, characterized by a still prevailing agricultural vocation, accommodates about 38000 lively farms producing a 40 % of the entire agriculture productivity of all Campania (di Gennaro, 2014).

2.2 Biomonitoring survey

2.2.1 Experimental design, bags preparation and exposure

The methodology applied follows a protocol of exposure in which devitalized moss *Hypnum cupressiforme* Hedw. was used to prepare sub-spherical moss bags that were exposed in the sites of interest for six weeks (Ares et al., 2012 modified). The exposure design was applied to five municipalities of Campania RIPS – Acerra (Ac), Casal di Principe (C), Giugliano (G), Maddaloni (M) and Teverola (T) - taking into account the inventory of known emissions to the atmosphere (for more details see Iodice et al., 2016) and the diffuse conurbation of the area in which agricultural (A) and urban/residential (U) sites are strictly intermingled. At each municipality, two scenarios and four sites were selected: two agricultural (A1 and A2) and two residential/urban (U1 and U2). To put in evidence the effects of vehicular traffic emissions on pollutant uptake by moss, at each site the bags were exposed in two sub-scenarios: directly on the street (S) and at green areas (G) as small gardens, at least 20 m far from the street, this criterion was adopted due to the landscape configuration. So, finally, eight different exposure points were selected at each municipality (e.g. for Acerra, AcA1S, AcA1G, AcA2S, AcA2G, AcU1S, AcU1G, AcU2S, AcU2G, see Figure 1 and Table S1), for a total of 40 exposure points.

The data refers to 39 measure points, since in one point (CA1S) the bags were lost during the exposure due to vandalism. For the bag preparation, samples of *Hypnum cupressiforme* Hedw. were collected in the pristine site of Sgonico (NE Italy) and processed according to the protocol by Ares et al. (2012). Moss aliquots (500 mg) were taken in round bags (moss weight/bag surface ratio 10 mg/cm^2) prepared with a nylon net (2 mm mesh), suspended on latticework by nylon strings and hanged at 4 m above ground level. Three bags were placed at each exposure point for metal analysis; additional bags (4 bags * 3 replicas) were placed at five agricultural sites (one for each municipality) for PAH analysis. All the bags were exposed for six weeks starting on March 2014. Samples of background moss were analyzed to assess baseline element (n = 8) and PAH (n = 3) contents.

2.2.2 Analytical procedures

Metals and metalloids

After the exposure the moss material removed from each nylon bag was oven dried at 40 °C, processed and analyzed separately. Moss was firstly milled and homogenized using a Retsch PM200 ball miller equipped of agate pockets. Then, for metals and metalloids analysis, 500 mg of pre and post-exposure moss samples were mineralized with ACS-grade HNO_3 for 1 h and, then, with aqua regia (ACS-grade $HCl–HNO_3$ in a volume ratio of 1:3) in a boiling water bath (95°C) for 1 h. Sample solutions were analyzed with a Perkin Elmer Elan 6000 ICP mass spectrometer. All concentrations were reported on a dry weight basis. Procedural blanks were usually below detection limits and M3 certified reference moss from the Finnish Forest Research Institute (Steinnes et al., 1997) was used for quality control. A total of 39 elements were analyzed (Al, As, B, Ba, Ca, Cd, Ce, Co, Cr, Cs, Cu, Fe, Hf, Hg, K, La, Li, Mg, Mn, Mo, Na, Nb, Ni, P, Pb, S, Sb, Sc, Se, Sn, Sr, Th, Ti, Tl, U, V, Y, Zn, Zr). For the majority of the elements the percentage of recovery ranged between 70 % and 110 %.

PAHs

For PAH analyses, moss samples (2 g obtained combining the 4 bags for each replica) were sonicated (Falc Sonicator) for two times, each in 25 mL of dichloromethane for 20 min each. The extracts were purified through activated silica gel and dried to a volume of 200 µL under a gentle nitrogen stream. Consecutively the samples were analyzed by GC–MSD (Agilent 5975C with a VF-17MS column) with helium as gas carrier at 1.3 mL min^{-1}. The oven temperature program started at 50 °C, increased with ramp rate 30 °C min^{-1}, to 350 °C and held for 9 min. All analyses were performed in selected ion monitoring (SIM). The concentrations of the following 20 PAHs, naphtalene (Naph), acenaphthene (Ace), fluorene (Flu), phenanthrene (Phen), anthracene (Ant), fluoranthene (Flt), pyrene (Pyr), benz[a]anthracene (B[a]A), chrysene (Chrys), benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[j]fluoranthene (B[j]F), benzo[a]pyrene (B[a]P), dibenz[a,h]anthracene (DB[a,h]A), indeno[1,2,3-c,d]pyrene (IP), benzo[g,h,i]perylene (B[g,h,i]P), dibenzo[a,i]pyrene (DB[a,i]P), dibenzo[a,h]pyrene (DB[a,h]P), dibenzo[a,e]pyrene (DB[a,e]P) and dibenzo[a,l]pyrene (DB[a,l]P), were quantified by multi-point calibration curves and labelled internal standards. For the quality control of the procedure, labelled PAHs (naphthalene D8, acenaphtene D10, phenanthrene D10, chrysene D12, perylene D12) were used as surrogates and the percentage recoveries (from 82 % to 120 %) included to correct the concentration of each compound. The minimum detectable PAH concentration was 1 ng g^{-1} d.w. for each compound.

2.2.3 Data analysis

Basic statistics were completed by using Microsoft Office Excel 2010, all the others were performed with STATISTICA StatSoft, 2008. The significance of element accumulation was evaluated by comparing pre-exposure to post-exposure values following Couto et al. (2004) as modified in Ares et al. (2015). The non-parametric Mann-Whitney U test was also performed both for testing the significance of accumulation and comparing agricultural (A) and urban (U) sites ($p < 0.05$). A Wilcoxon matched pairs test was used to compare the moss exposed at street (S) sites with those exposed in corresponding green ones (G) ($p < 0.05$).

3. Results and Discussion

3.1 Chemical elements

The concentrations of 39 analyzed elements (mean values ± standard deviations) accumulated by moss in the 39 selected sites are reported in supplemental Table S1. A significant accumulation ($p < 0.05$) of alkaline and alkaline-earth metals (i.e. Ba, Ca, Cs, Li, Mg, Sr) was observed in 26 sites out of 39; no accumulation occurred for K and Na. As for the transition metals, moss accumulation was observed on average in 26 sites; we found no accumulation for Cd and Hg; Co, Hf, V were accumulated in less than half sites, whereas Cu, Cr, Fe, Mn, Mo, Nb, Zn, Zr were significantly accumulated in almost all sites. The lanthanoides and actinoides, with the exception of Sc (never accumulated) and U (found in less than half of the sites), were accumulated in at least 33 sites. Among the other elements a significant accumulation of Pb and Sb was observed in all sites, while Al and Sn accumulated in 25 and 29 sites, respectively; no accumulation was observed for P. The most of the elements were significantly accumulated in the following sites: TA1S and MA1S (respectively 32 and 31 elements), GA2S and TA1V (30), all agricultural sites. The minimum number of elements accumulated was recorded at MU1V (15), MU2V (17), all urban sites.

The elements most accumulated by mosses exposed in A sites with respect to the those exposed in U sites were: Al, Ce, Cs, Hf, La, Mg ,Mn, Nb, Pb, Sr, Th ,Ti, Tl, U, Y, Zn, Zr ($p < 0.05$). High amount of Al in A sites suggests the resuspension of soil particles in the ambient air. The salts of Mg, Mn and Zn are largely employed as fertilizers while uranium could be found in phosphate fertilizer of mineral origins when not appropriately purified (Jacques et al., 2008). The presence of Rare Earth Elements in agricultural sites and their significant correlation with Al suggest a derivation from soil (see Table S2); in fact, it was reported that mixtures of REEs in fertilizers are nowadays widely used in agriculture to improve crop nutrition (Tyler, 2004). Moreover, REEs are increasingly used in modern technologies such as electronic devices, which are among the wastes frequently and illegally abandoned in the fields. A similar correlation with Al (already seen for REEs) was found also for Ti and Tl; in particular, the correlation with Ti could be explained by the high flow of heavy-duty vehicles, which enhance the resuspension of soil dust particles (Adamo et al., 2011). Other well recognized sources of Ti, in the form of TiO_2 nanoparticles, are sunscreens, cosmetics and industrial applications (Luo et al., 2014); therefore the disposal of materials reach in Ti could justify its uptake in moss transplants both in urban/residential and in agricultural lands. A notable increment of Ti in the exposed moss was also observed by Adamo et al. (2011), in two biomonitoring surveys carried out in 1999 and 2006 in the Naples urban area. Hafnium and Sr have their application in the old and modern technologies (Greenfield at al., 2013); in particular, Hf is used in manufacturing of processors, while strontium was used as oxide for cathode ray tubes of old generation color TV (Mear et al., 2005), and as salt in fireworks (Shimizu, 1998) largely exploded over the investigated rural areas. We also suppose the derivation of these two elements from the deterioration of abandoned electronic devices, especially present in proximity of agricultural areas, as previously hypothesized for REEs. The finding that Pb is mostly accumulated by moss in A sites was already observed (Iodice et al., 2016), and attributed to heavy-duty vehicular traffic in the area. The presence of Nb in the A sites can be related to the intense air traffic. Niobium is used in high purity alloys with iron, nickel and cobalt for the realization of parts of jet engines, rocket parts and structures resistant to high temperatures (Perepezko, 2009); the investigated area is indeed located between two airports (Grazzanise Military Airport and Capodichino Civil Airport) with air traffic particularly routed over uninhabited sites, as the A sites are.

The exposure in sub-scenarios (G and S) showed that mosses placed at S sites always accumulated higher element amounts than those at G sites; a significant difference ($p < 0.05$) occurred for 17 of the investigated elements (Al, Ca, Ce, Co, Cs, Cu, Fe, La, Mn, Mo, Nb, Sn, Sr, Ti, U, Y, Zr). As a matter of fact, mosses were able to discriminate between front road sites (S) and matching green ones (G), far from each other not more than 20 m; this finding indicates a high sensitivity of moss-bag approach.

Two PCAs (principal component analysis) were performed: the first averaging all values of element content in mosses exposed at the agricultural (A) and at the urban (U) sites; the second averaging all values of element content in mosses exposed at street (S) and at matching green (G) sites, in order to compare moss uptakes in these two scenarios and sub-scenarios. The PCA showing A and U sites (Figure 2a) indicates that the two classes are clearly divided along the Factor 1 (54 % of the total variance), with all U sites nearer to the pre-exposure values (T0) than A sites, the latter showing higher element accumulation. Although some site diversification also occurs along the Factor 2, this only explains the 13 % of the total variance. The projection of the variables are shown in the Figure 2b.

Most of the elements concurring to the variance of the Factor 2 were those lost or not accumulated during the exposure, all the other elements explaining the variance of the Factor 1. The PCA indicates Teverola agricultural sites as those showing the highest element load and Casal di Principe and Maddaloni urban sites as those with the lowest accumulation. Within each scenario (A *vs* U) Teverola is the most impacted municipality and Casal di Principe the less impacted. The PCA comparing G *vs* S sites is reported in Figure 3. The Factor 1 explains 55 % of the total variance and is related to those elements accumulated during the exposure; the Factor 2 accounts for about 13 % of total variance. With the exception of some overlapping, G sites are separated from the S sites along the Factor 1, with the latter at higher distance from T0 than the former. This indicates a higher accumulation in mosses exposed in front road sites, irrespectively of the scenario (U or A). As for the previous PCA, Teverola is the most impacted site while Casal di Principe the least, and this is true for both sub-scenarios (G and S).

The Figure 4 shows the total element accumulation observed at each site obtained by summing the concentrations normalized to the maximum value for each element. Although the confidential intervals (CIs) of the two scenarios are different only for Casal di Principe and Teverola, agricultural sites always showed a total element content higher than urban sites (Figure 4a).

When we compare the two sub-scenarios (G *vs* S), G sites generally show lower moss uptake than S sites and this difference is particularly evident for Maddaloni (Figure 4b). If we consider the total accumulation (all scenarios) at each town (Figure 4c), Teverola shows the highest content of element, although the differences among municipalities are not substantial (i.e., overlapping of CIs), suggesting a homogeneous element deposition over the entire study area.

Tretiach et al. (2011) applied a similar exposure design, comparing moss uptake (using oven devitalised *H. cupressiforme* transplanted in bags) between residential versus residential/industrial sites, and roadsides versus green sites. Their findings indicated a clear distinction between residential and residential/industrial scenarios, while the two sub-scenarios (roadsides *vs* green sites) were less clearly partitioned. In our experimental design we also found a clear distinction between the two scenarios (A *vs* U), especially evident for Casal di Principe and Teverola (Figure 4a). Considering sub-scenarios (G *vs* S), we generally found higher accumulation in mosses exposed in front road sites, particularly evident for Maddaloni.

The current results show that moss uptakes are always lower than those recorded in a previous survey carried out in the Naples urban area (Adamo et al., 2007) with the exception of Mg, showing higher accumulation in the agricultural scenario of all five towns. This outcome is probably due to the large use of Mg as fertilizer especially in the form of Epsom salt ($MgSO_4 \cdot 7H_2O$). This salt is employed to correct the deficiency of Mg in the agricultural soils and is essential for potatoes and tomatoes (Bolton, 1973; Sainju et al., 2003) that are among the main crops from our study area (INEA, 2011).

3.2 PAHs

The total PAH concentration in the moss before exposure was 59 ng g^{-1} d.w., increasing after exposure in all five municipalities, with a post-exposure total PAH concentrations ranging from 72 (Teverola) to 95 ng g^{-1} d.w. (Maddaloni and Acerra). Grouping the PAHs according to the ring number, before the exposure, the 2-, 3- and 4-ring PAHs (Figure 5), represented the 24, 32 and 40 %

of the total content, respectively. Phenanthrene (15.7 ng g^{-1} d.w.), Naph (14.3 ng g^{-1} d.w.) and B[a]A (14.1 ng g^{-1} d.w.), were the most represented compounds in the unexposed moss (Table 1).

After exposure, the average amount of accumulated PAHs (i.e. post- minus pre-exposure value) over all sites, was still prevalently characterized by the 4-ring PAHs (51 % of the total PAHs), followed by the 5-ring PAHs (24 % of the total PAHs) that were below the detection limit in the unexposed moss. The higher percentage of low molecular weight PAHs in unexposed than in post-exposed moss was confirmed in previous studies (Vingiani et al., 2015). The most accumulated compounds were, on average over all the sites, Chrys (8.6 ng g^{-1} d.w.), Flt (7.3 ng g^{-1} d.w.) and Pyr (6.5 ng g^{-1} d.w.). It should be noted that the B[a]P, the most extensively measured PAH around the world due to its proved carcinogenic property, is absent in the moss before exposure and is absent or very low also after exposure in all municipalities (Table 1).

Few data are available about air PAH monitoring by *Hypnum cupressiforme* moss-bags: in the urban area of Belgrade PAH concentrations in moss-bags ranged from 80 to 250 ng g^{-1} d.w. (Vuković et al., 2015), whereas in London and Naples (Vingiani et al., 2015) the average total PAH accumulation in *Hypnum cupressiforme* exposed was one order of magnitude higher than the uptakes measured in the present study. Comparing the present findings to the results previously reported for a street canyon in the urban area of Naples (De Nicola et al., 2013), we recorded higher accumulation in the canyon for all PAHs, excepted IP with similar values, and Ant and DB[a,h]A with higher values in the present test. In a recent study (Monaco et al., 2015), the "Litorale Domizio-Agro Aversano", where the investigated agricultural sites are located, was monitored for soil PAH concentrations, being the soils of this area widely affected by storage of waste, or subject to illicit dumping of unknown material. Similarly to what observed in the exposed moss, also in the soils (two of which exactly located in the towns of Giugliano and Teverola), pyrene and chrysene were highly represented respect to the total PAH content. Even if we cannot exclude that part of PAH uptake in moss may derive from particle soil resuspension, the different environmental matrices (soil and moss) and their different exposure times should be considered when discussing these data (Augusto et al., 2010).

The levels of single PAHs can be used as markers suggesting PAH origin sources (Ravindra et al., 2008 and reference therein): dominance of Chrys, as found in the PAH profile of the moss-bags, was suggested in some studies as a marker of coal and wood combustion (Ravindra et al., 2008; Dvorská et al., 2011), confirmed also by the diagnostic ratio IP/(IP + B[g,h,i]P) higher than 0.5 in the exposed moss (Yunker et al., 2002; Tobiszewski and Namieśnik, 2012). Pyr and Flt resulted high in emissions from incineration and oil combustion; moreover other studies reported that the diesel exhausts are enriched in Flt, Pyr and Chrys respect to gasoline exhausts (Larsen and Baker, 2003). The ratio Flt/(Flt + Pyr) equal to 0.5 seems to indicate also the presence of vehicular emissions (Yunker et al., 2002) in the investigated area. Flt and Pyr are reported among the dominant compounds in the PAH profiles of *Hypnum* moss-bags exposed in urban areas (De Nicola et al., 2013; Vuković et al., 2015). However, it is noteworthy to remember that the ratios are often unable to resolve sources with sufficient accuracy (Singh et al., 2008), especially in the investigated area where agricultural and urban sites, and their pollutant emission sources, are strictly mixed.

4. Conclusions

Results of the present survey indicate *Hypnum cupressiforme* moss bags as a very sensitive tool to detect airborne element and PAH inputs in a landscape characterized by a jeopardized structure in which agricultural and urban/residential sites are strictly mixed together. Particularly, through the exposure scheme adopted for elements, we were able to discriminate different pollution levels in agricultural and urban scenarios and green and street sub-scenarios. In general, agricultural sites showed higher moss uptake than urban ones, as well as moss suspended at street sites, directly facing the vehicular traffic. On the basis of moss uptake, the whole study area is homogeneously polluted. Agricultural practices and road traffic appear to be the major diffuse pollution sources over the whole study area, both producing polluted particulate matter that mosses are able to entrap. The impact of agricultural practices, indicated by our survey at a regional scale, seems in line with a global trend; agriculture indeed, has been recently reported as the leading source of particulate matter (specifically $PM_{2.5}$) in Europe, with a contribution of 40 % or higher in many European countries (Lelieveld et al., 2015). Such a detailed information, up to a very small land scale, would never be possible by using monitoring stations, also considering the large set of pollutants here analyzed (i.e., 39 chemical elements and 20 PAHs). Therefore, moss bags should be considered as a valid candidate for a qualitative appraisal of atmospheric pollution for regulatory purposes.

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Tables

Table 1. Concentrations of each PAH (ng g^{-1}, mean ± s.d. n = 3) in the moss before (T0) and after exposure at one agricultural site for each studied municipality. The concentration of total PAHs (∑PAHs) is also reported. M = Maddaloni; C = Casal di Principe; T = Teverola; Ac = Acerra; G = Giugliano.

	rings	**T0**	**M**	**C**	**T**	**Ac**	**G**
Naph	2	14.3±2.1	13.4±7.0	16.5±0.6	17.5±1.6	18.7±4.7	18.9±2.5
Ace	3	<dl	3.1±0.7	2.2±1.5	3.1±0.1	2.1±1.4	1.6±1.0
Flu	3	2.0±2.5	5.9±3.3	2.2±3.0	3.4±0.8	4.7±0.8	2.3±1.9
Phen	3	15.7±2.4	16.6±0.9	13.9±1.2	12.4±0.9	15.7±2.2	13.1±1.0
Ant	3	1.5±0.2	6.3±10.0	<dl	<dl	<dl	<dl
Flt	4	4.4±0.5	10.8±0.7	13.0±0.6	10.0±0.8	12.7±0.9	12.3±1.4
Pyr	4	4.0±0.5	9.4±0.8	12.2±0.8	8.6±0.7	11.3±0.3	11.0±1.0
B[a]A	4	14.1±8.2	<dl	<dl	<dl	1.5±0.1	1.0±0.4
Chrys	4	1.1±0.5	11.0±0.2	10.2±0.3	6.1±0.4	11.9±0.4	9.2±1.7
B[b]F	5	<dl	5.2±0.5	5.4±0.2	3.8±0.3	6.0±0.1	4.7±0.4
B[k]F	5	<dl	2.9±2.4	3.1±0.2	1.4±0.2	1.8±0.3	1.4±0.1
B[j]F	5	<dl	3.1±1.6	2.5±0.3	1.6±0.1	2.4±0.1	2.0±0.2
B[a]P	5	<dl	1.7±2.0	0.9±0.6	<dl	<dl	1.2±1.2
DB[a,h]A	5	<dl	2.4±3.3	<dl	<dl	<dl	<dl
IP	6	0.8±0.5	2.2±1.5	2.3±1.5	2.6±0.1	1.3±1.4	2.6±0.1
B[g,h,i]P	6	1.5±0.1	1.5±1.7	2.4±1.7	1.9±1.2	3.0±0.1	1.9±1.2
DB[a,i]P	6	<dl	<dl	<dl	<dl	<dl	<dl
DB[a,h]P	6	<dl	<dl	<dl	<dl	<dl	<dl
DB[a,e]P	6	<dl	<dl	<dl	<dl	<dl	<dl
DB[a,l]P	6	<dl	<dl	<dl	<dl	<dl	<dl
∑PAHs		59.4±6.7	95.3±18.6	86.7±6.1	72.2±5.5	94.9±4.9	83.3±6.2

<dl: *below detection limit*

Figures

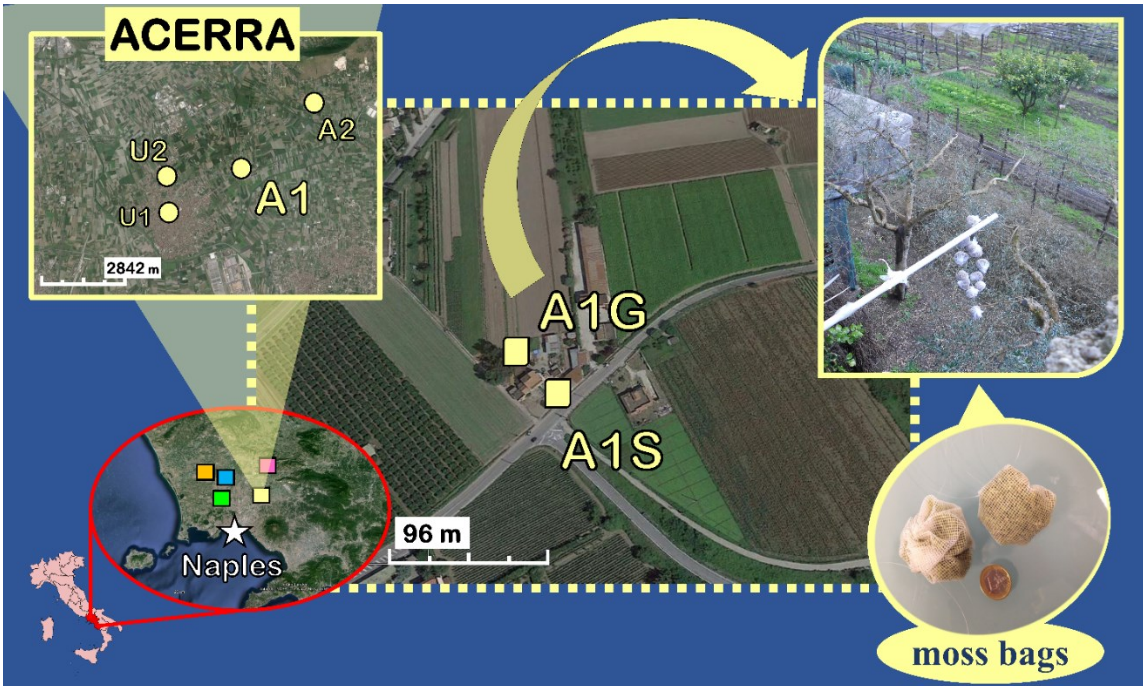

Figure 1. Outline of the moss bag exposure design in the study area with a shoot up on Acerra agricultural (A1 and A2) and urban/residential (U1 and U2) sites. The arrangement of moss bags in green (G) and street (S) exposure points at the A1 site is also shown.

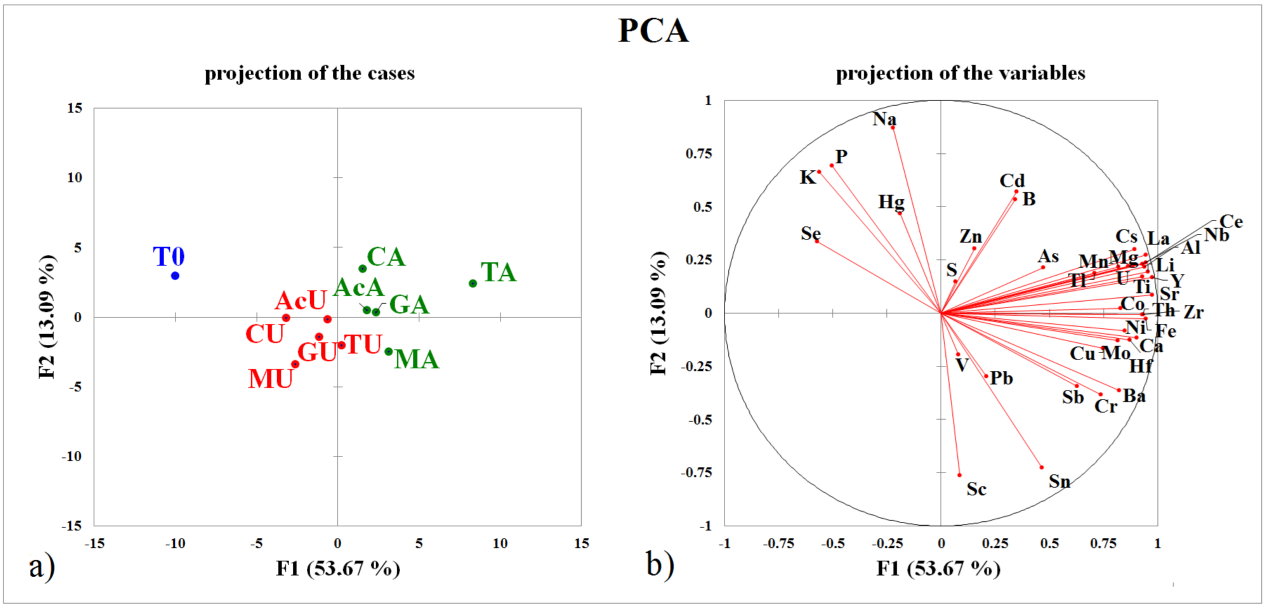

Figure 2. PCA considering agricultural (A) and urban (U) scenarios at each municipality: a) projection of the cases (n = 11); b) projection of the variables (n = 39). See the text for the label codes.

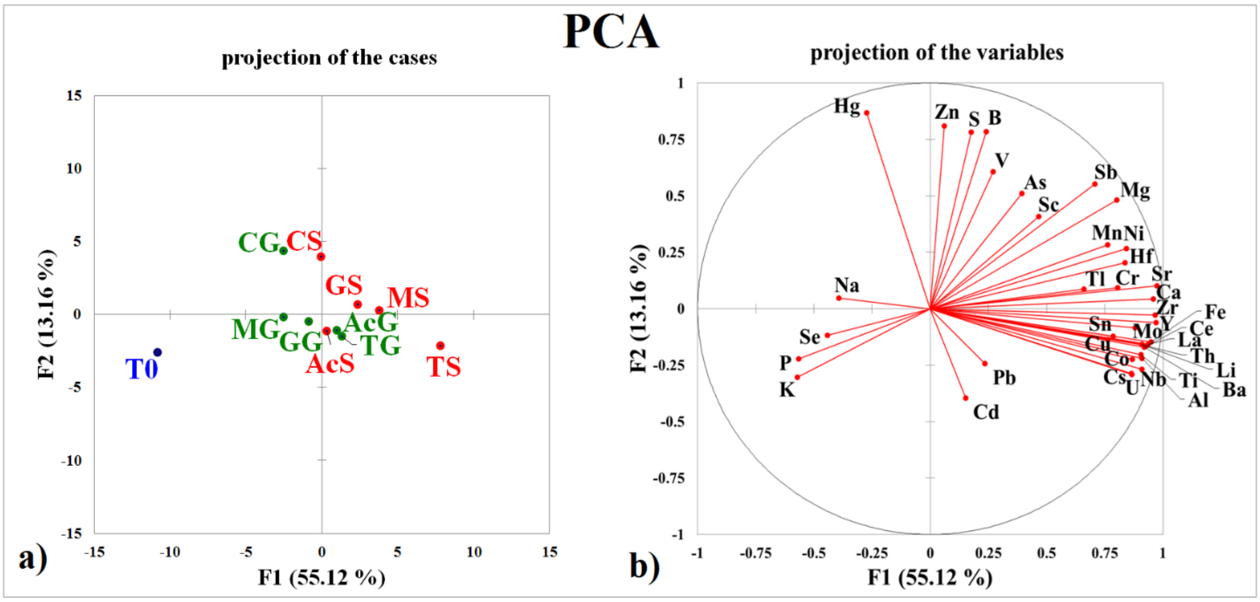

Figure 3. PCA considering green (G) and street (S) sub-scenarios at each municipality: a) projection of the cases (n = 11); b) projection of the variables (n = 39). See text for label codes.

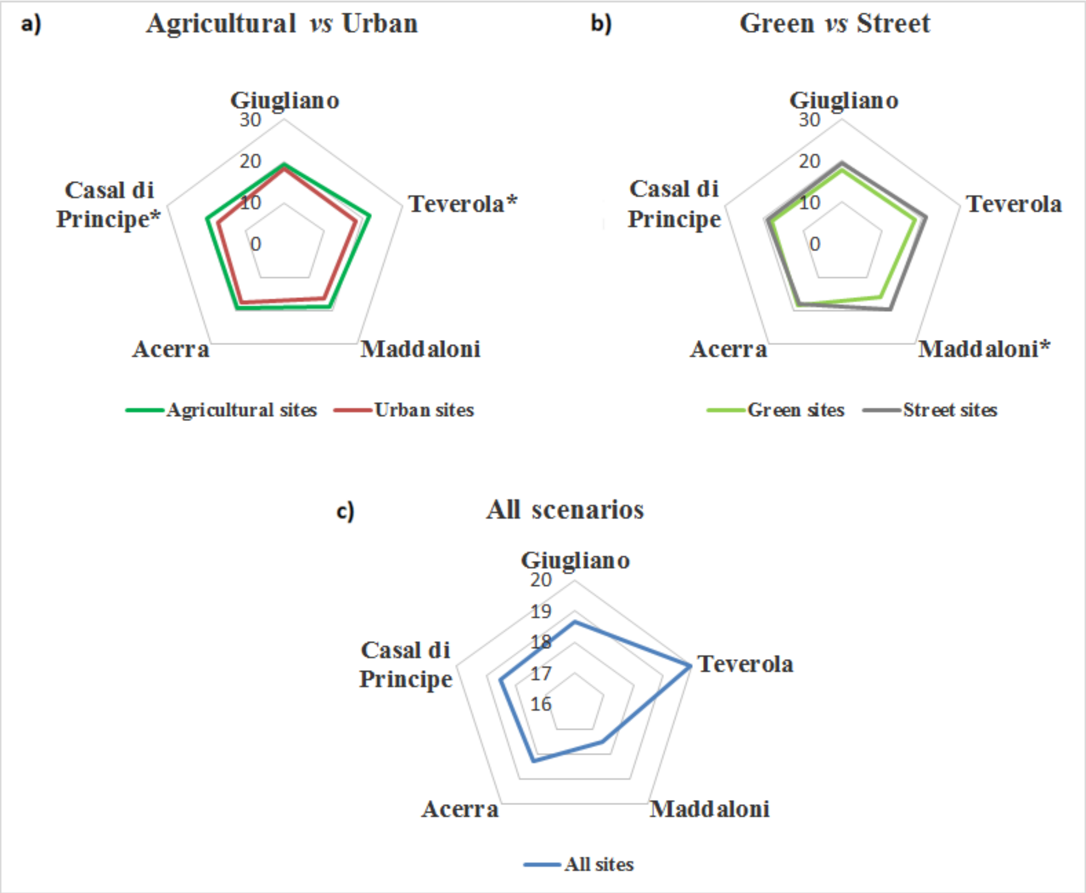

Figure 4. Total element accumulation (sum of the normalized values) observed at each municipality. a) agricultural (A) versus urban (U) sites; b) green (G) versus street (S) sites; c) data from all scenarios. Asterisks indicate that confidence intervals do not overlap.

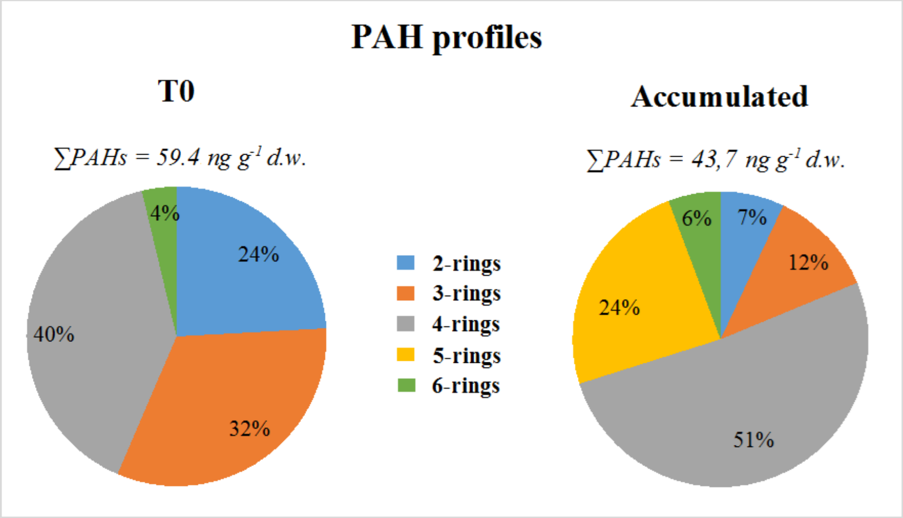

Figure 5. Profiles of PAHs grouped by number of rings, as percentage of total PAHs, in pre-exposure moss (T0) and accumulated in the exposed moss bags (mean over all sites).

Graphical abstract

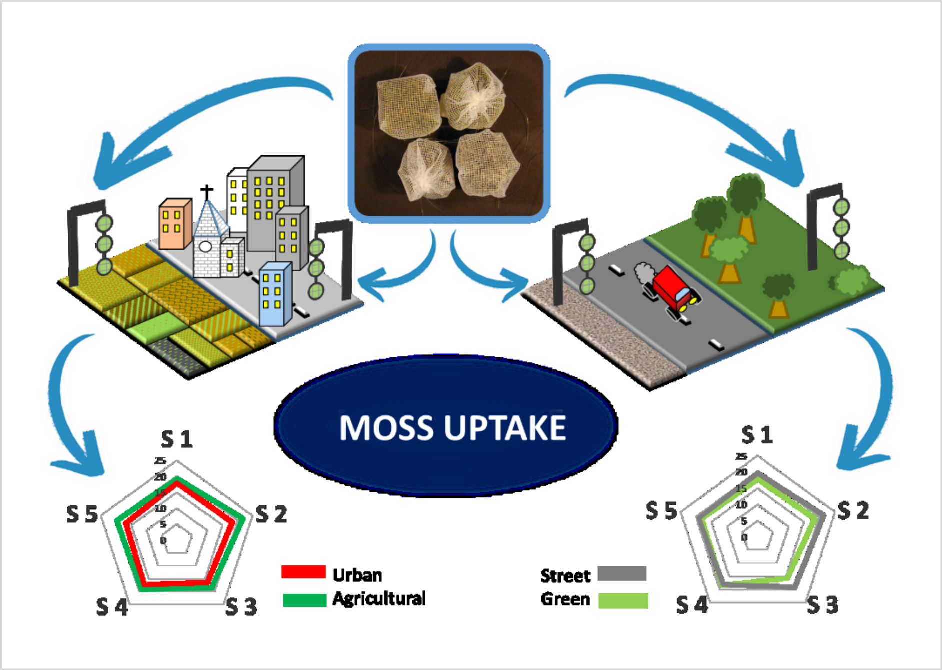

Table S1. Element concentrations (mg kg^{-1}, mean values ± standard deviations, n = 3) of 39 elements in moss exposed at 39 sites. T0 = pre-exposure values (n = 8). See the text for the label codes.

	T0	**GA1G**	**GA1S**	**GA2G**	**GA2S**	**GU1G**	**GU1S**	**GU2G**	**GU2S**	**TA1G**	**TA1S**	**TA2G**	**TA2S**	**TU1G**	**TU1S**	**TU2G**	**TU2S**	**MA1G**	**MA1S**	**MA2G**
Al	*300 ± 76*	500 ± 100	600 ± 100	667 ± 115	533 ± 115	333 ± 153	533 ± 58	533 ± 115	467 ± 58	733 ± 153	1.533 ± 321	533 ± 58	433 ± 153	400 ± 100	467 ± 115	367 ± 58	633 ± 58	467 ± 58	733 ± 208	433 ± 58
As	*0.08 ± 0.05*	0.20 ± 0.23	0.05 ± 0.00	0.10 ± 0.06	2.07 ± 0.12	0.07 ± 0.00	0.12 ± 0.06	0.10 ± 0.06	0.20 ± 0.23	0.07 ± 0.00	0.38 ± 0.26	0.28 ± 0.20	0.43 ± 0.12	0.28 ± 0.26	0.17 ± 0.17	0.23 ± 0.29	0.10 ± 0.06	0.25 ± 0.21	0.60 ± 0.40	0.08 ± 0.00
B	3.00 ± 0.00	3.67 ± 0.58	4.33 ± 1.15	3.67 ± 0.58	4.33 ± 0.58	4.00 ± 0.00	5.00 ± 0.00	3.00 ± 0.00	3.00 ± 1.00	3.67 ± 0.58	7.33 ± 1.15	3.00 ± 1.00	3.67 ± 0.58	3.33 ± 0.58	4.00 ± 0.00	3.33 ± 0.58	3.00 ± 0.00	3.33 ± 0.58	4.00 ± 1.00	3.67 ± 0.58
Ba	14.38 ± 0.82	16.70 ± 1.21	18.67 ± 1.71	18.03 ± 1.11	18.63 ± 0.15	16.53 ± 0.49	19.23 ± 0.95	17.67 ± 0.50	18.47 ± 0.68	18.97 ± 0.35	26.57 ± 3.02	16.63 ± 0.31	16.00 ± 1.04	16.40 ± 1.01	17.20 ± 0.26	24.80 ± 1.14	18.43 ± 0.06	17.77 ± 0.95	21.17 ± 1.96	15.60 ± 1.15
Ca	*3638 ± 92*	4833 ± 208	7533 ± 1.002	4867 ± 208	5200 ± 100	5033 ± 252	5800 ± 100	5533 ± 551	5733 ± 153	6033 ± 289	12167 ± 2031	4700 ± 100	4467 ± 231	4433 ± 404	5000 ± 173	4767 ± 252	5300 ± 100	5200 ± 200	8367 ± 902	4533 ± 58
Cd	*0.08 ± 0.02*	0.07 ± 0.01	0.09 ± 0.01	0.10 ± 0.03	0.10 ± 0.02	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.02	0.09 ± 0.01	0.09 ± 0.02	0.09 ± 0.01	0.09 ± 0.00	0.09 ± 0.02	0.08 ± 0.03	0.07 ± 0.00	0.11 ± 0.01	0.09 ± 0.01	0.08 ± 0.02	0.08 ± 0.01	0.06 ± 0.01
Ce	*0.29 ± 0.02*	0.66 ± 0.08	0.80 ± 0.13	0.95 ± 0.12	0.80 ± 0.12	0.40 ± 0.03	0.62 ± 0.05	0.66 ± 0.06	0.59 ± 0.04	1.14 ± 0.17	2.95 ± 0.87	0.60 ± 0.02	0.66 ± 0.14	0.52 ± 0.06	0.59 ± 0.08	0.47 ± 0.04	0.82 ± 0.08	0.80 ± 0.04	1.20 ± 0.24	0.55 ± 0.04
Co	*0.12 ± 0.02*	0.13 ± 0.03	0.18 ± 0.04	0.16 ± 0.04	0.15 ± 0.02	0.12 ± 0.01	0.19 ± 0.03	0.17 ± 0.02	0.17 ± 0.05	0.22 ± 0.01	0.40 ± 0.08	0.18 ± 0.03	0.16 ± 0.07	0.15 ± 0.03	0.19 ± 0.05	0.28 ± 0.04	0.22 ± 0.02	0.17 ± 0.03	0.22 ± 0.03	0.10 ± 0.02
Cr	*2.25 ± 0.20*	2.57 ± 0.31	2.47 ± 0.21	2.33 ± 0.12	4.97 ± 0.51	2.67 ± 0.42	2.87 ± 0.31	3.13 ± 0.40	2.93 ± 0.31	3.43 ± 0.35	3.47 ± 0.15	2.90 ± 0.52	2.77 ± 0.40	2.67 ± 0.31	2.87 ± 0.23	4.17 ± 0.25	3.13 ± 0.21	2.83 ± 0.15	3.07 ± 0.40	2.60 ± 0.17
Cs	*0.07 ± 0.01*	0.11 ± 0.01	0.13 ± 0.02	0.16 ± 0.01	0.13 ± 0.00	0.08 ± 0.00	0.10 ± 0.00	0.10 ± 0.01	0.11 ± 0.00	0.19 ± 0.03	0.50 ± 0.11	0.10 ± 0.01	0.11 ± 0.02	0.09 ± 0.00	0.11 ± 0.02	0.08 ± 0.00	0.12 ± 0.00	0.13 ± 0.01	0.18 ± 0.03	0.10 ± 0.01
Cu	*4.77 ± 0.28*	5.88 ± 0.09	5.81 ± 0.24	6.87 ± 0.18	13.34 ± 1.82	6.09 ± 0.69	8.14 ± 0.49	6.56 ± 0.18	7.63 ± 0.60	8.08 ± 1.10	8.57 ± 0.62	6.23 ± 0.78	6.14 ± 0.68	5.88 ± 0.12	7.26 ± 0.73	6.35 ± 0.43	7.20 ± 0.16	6.87 ± 0.55	13.07 ± 1.84	6.09 ± 0.30
Fe	*404 ± 15.98*	527 ± 30.55	590 ± 45.83	603 ± 35.12	577 ± 11.55	460 ± 20.00	580 ± 36.06	640 ± 91.65	613 ± 25.17	727 ± 98.66	1.160 ± 199.25	517 ± 15.28	490 ± 72.11	490 ± 20.00	567 ± 55.08	883 ± 105.04	600 ± 10.00	577 ± 55.08	820 ± 110.00	477 ± 35.12
Hf	*0.01 ± 0.00*	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.00	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.03 ± 0.01	0.01 ± 0.00
Hg	*48 ± 16*	50 ± 3	39 ± 3	49 ± 12	49 ± 5	47 ± 7	51 ± 10	36 ± 8	44 ± 12	48 ± 4	41 ± 2	45 ± 6	38 ± 14	48 ± 12	49 ± 5	45 ± 3	40 ± 13	46 ± 2	46 ± 6	38 ± 6
K	*3138 ± 52*	1933 ± 153	1733 ± 231	2000 ± 200	1967 ± 58	2400 ± 265	1967 ± 58	2033 ± 153	2100 ± 100	2133 ± 115	3000 ± 100	2267 ± 153	2000 ± 173	2233 ± 58	2267 ± 115	2200 ± 173	2200 ± 100	1767 ± 252	1633 ± 153	1567 ± 153
La	*0.14 ± 0.03*	0.33 ± 0.05	0.44 ± 0.09	0.50 ± 0.07	0.41 ± 0.02	0.20 ± 0.02	0.29 ± 0.04	0.28 ± 0.03	0.31 ± 0.02	0.58 ± 0.09	1.44 ± 0.34	0.26 ± 0.01	0.31 ± 0.06	0.24 ± 0.02	0.29 ± 0.03	0.22 ± 0.02	0.38 ± 0.03	0.39 ± 0.06	0.59 ± 0.10	0.29 ± 0.03
Li	0.19 ± 0.02	0.36 ± 0.06	0.36 ± 0.04	0.38 ± 0.08	0.32 ± 0.13	0.28 ± 0.01	0.30 ± 0.03	0.35 ± 0.04	0.35 ± 0.03	0.55 ± 0.10	0.98 ± 0.25	0.27 ± 0.05	0.31 ± 0.07	0.34 ± 0.02	0.45 ± 0.07	0.23 ± 0.02	0.39 ± 0.02	0.33 ± 0.07	0.43 ± 0.06	0.35 ± 0.04
Mg	*813 ± 18*	953 ± 12	1030 ± 30	1000 ± 30	1003 ± 35	957 ± 23	1107 ± 47	987 ± 21	1023 ± 6	1110 ± 36	1420 ± 111	1010 ± 10	987 ± 45	953 ± 58	1010 ± 26	940 ± 40	990 ± 26	1017 ± 49	1070 ± 46	970 ± 26
Mn	*10 ± 1*	14 ± 1	15 ± 1	18 ± 1	17 ± 1	12 ± 1	14 ± 1	16 ± 1	15 ± 1	19 ± 2	37 ± 7	19 ± 4	14 ± 1	13 ± 1	15 ± 1	16 ± 1	17 ± 3	17 ± 1	23 ± 2	14 ± 1
Mo	*0.22 ± 0.02*	0.28 ± 0.03	0.31 ± 0.03	0.41 ± 0.02	0.41 ± 0.06	0.29 ± 0.02	0.33 ± 0.04	0.32 ± 0.02	0.35 ± 0.03	0.36 ± 0.05	0.45 ± 0.04	0.29 ± 0.05	0.40 ± 0.01	0.28 ± 0.03	0.35 ± 0.05	0.54 ± 0.06	0.34 ± 0.02	0.30 ± 0.04	0.46 ± 0.06	0.26 ± 0.04
Na	*1863 ± 58*	1353 ± 29	1260 ± 174	1390 ± 90	1467 ± 40	1677 ± 183	1473 ± 29	1307 ± 166	1423 ± 91	1527 ± 42	2057 ± 21	1420 ± 96	1430 ± 128	1533 ± 81	1653 ± 65	1367 ± 85	1520 ± 96	1360 ± 181	1220 ± 114	1243 ± 49
Nb	*0.02 ± 0.00*	0.11 ± 0.01	0.12 ± 0.02	0.18 ± 0.02	0.16 ± 0.03	0.04 ± 0.01	0.06 ± 0.02	0.07 ± 0.01	0.09 ± 0.01	0.17 ± 0.03	0.56 ± 0.15	0.08 ± 0.02	0.10 ± 0.01	0.06 ± 0.00	0.07 ± 0.02	0.05 ± 0.02	0.12 ± 0.02	0.12 ± 0.02	0.21 ± 0.04	0.10 ± 0.01
Ni	*0.84 ± 0.11*	1.00 ± 0.20	0.97 ± 0.12	1.33 ± 0.06	1.03 ± 0.06	0.90 ± 0.10	1.37 ± 0.21	1.27 ± 0.29	1.13 ± 0.06	1.13 ± 0.31	1.53 ± 0.06	1.17 ± 0.29	1.13 ± 0.15	0.97 ± 0.06	1.13 ± 0.15	1.20 ± 0.26	1.10 ± 0.20	1.00 ± 0.10	1.00 ± 0.10	0.73 ± 0.15
P	*1.118 ± 24*	820 ± 10	853 ± 40	893 ± 31	883 ± 32	1.000 ± 44	930 ± 79	917 ± 50	910 ± 35	917 ± 40	967 ± 31	930 ± 53	897 ± 65	883 ± 42	957 ± 49	910 ± 62	903 ± 32	860 ± 20	850 ± 20	760 ± 44
Pb	*0.58 ± 0.03*	1.99 ± 0.26	1.91 ± 0.44	3.41 ± 0.13	3.47 ± 0.20	1.60 ± 0.10	2.03 ± 0.09	2.83 ± 0.71	1.83 ± 0.09	5.17 ± 0.35	6.82 ± 0.93	4.81 ± 0.49	5.37 ± 1.18	1.74 ± 0.21	3.02 ± 0.41	81.21 ± 18.37	3.77 ± 0.77	4.61 ± 0.71	5.59 ± 1.20	3.14 ± 0.20
S	*513 ± 314*	217 ± 115	1.067 ± 252	867 ± 416	1367 ± 231	2333 ± 462	2267 ± 321	2100 ± 625	1400 ± 625	1333 ± 252	1367 ± 153	933 ± 321	700 ± 200	933 ± 115	1500 ± 200	967 ± 635	733 ± 153	467 ± 306	100 ± 0	400 ± 265
Sb	*0.36 ± 0.04*	0.82 ± 0.08	1.15 ± 0.37	0.65 ± 0.06	0.59 ± 0.02	0.73 ± 0.06	0.73 ± 0.04	0.68 ± 0.08	0.81 ± 0.09	0.76 ± 0.02	0.84 ± 0.16	0.77 ± 0.09	0.66 ± 0.13	0.86 ± 0.19	1.11 ± 0.19	0.75 ± 0.12	0.68 ± 0.06	0.72 ± 0.01	1.34 ± 0.22	0.97 ± 0.24
Sc	*0.33 ± 0.14*	0.20 ± 0.05	0.37 ± 0.06	0.40 ± 0.05	0.33 ± 0.06	0.30 ± 0.05	0.40 ± 0.05	0.37 ± 0.06	0.33 ± 0.06	0.37 ± 0.06	0.33 ± 0.06	0.37 ± 0.06	0.30 ± 0.08	0.37 ± 0.06	0.40 ± 0.08	0.33 ± 0.06	0.43 ± 0.06	0.40 ± 0.05	0.40 ± 0.08	0.33 ± 0.06
Se	*0.44 ± 0.07*	0.37 ± 0.06	0.63 ± 0.21	0.40 ± 0.30	0.27 ± 0.06	0.27 ± 0.06	0.43 ± 0.12	0.47 ± 0.12	0.23 ± 0.06	0.27 ± 0.06	0.23 ± 0.15	0.10 ± 0.06	0.30 ± 0.10	0.43 ± 0.21	0.27 ± 0.06	0.27 ± 0.06	0.18 ± 0.10	0.17 ± 0.17	0.17 ± 0.06	0.17 ± 0.17
Sn	*0.27 ± 0.02*	0.31 ± 0.07	0.43 ± 0.05	0.42 ± 0.04	0.45 ± 0.03	0.40 ± 0.05	0.71 ± 0.12	0.41 ± 0.03	0.54 ± 0.07	0.48 ± 0.04	0.48 ± 0.02	0.37 ± 0.03	0.34 ± 0.09	0.43 ± 0.01	0.58 ± 0.05	0.38 ± 0.06	0.53 ± 0.04	0.45 ± 0.04	0.94 ± 0.19	0.32 ± 0.10
Sr	*8.10 ± 0.21*	10.60 ± 0.46	13.13 ± 0.91	12.10 ± 0.26	12.40 ± 0.36	10.47 ± 0.49	12.93 ± 0.15	11.43 ± 0.75	12.10 ± 0.44	13.97 ± 0.40	23.77 ± 2.97	11.63 ± 0.15	11.33 ± 0.46	10.77 ± 0.67	11.37 ± 0.42	11.07 ± 0.70	12.27 ± 0.59	11.87 ± 0.67	15.33 ± 1.56	10.83 ± 0.12
Th	*0.04 ± 0.01*	0.08 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.04 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.00	0.14 ± 0.01	0.11 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.06 ± 0.00	0.07 ± 0.00	0.06 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.12 ± 0.02	0.07 ± 0.00
Ti	*6.50 ± 0.53*	12.33 ± 1.53	13.67 ± 2.31	16.67 ± 1.15	14.00 ± 1.00	8.33 ± 1.15	10.67 ± 1.15	11.67 ± 0.58	11.33 ± 1.15	19.33 ± 3.21	45.33 ± 9.24	10.67 ± 0.58	11.33 ± 2.31	9.67 ± 1.15	12.00 ± 2.65	9.33 ± 0.58	14.67 ± 0.58	14.67 ± 1.53	22.67 ± 4.51	12.00 ± 1.00
Tl	*0.01 ± 0.00*	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.04 ± 0.00	0.07 ± 0.01	0.02 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
U	*0.02 ± 0.01*	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.00	0.02 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.12 ± 0.03	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.01	0.04 ± 0.00	0.04 ± 0.01	0.06 ± 0.01	0.03 ± 0.00
V	*2.38 ± 1.51*	3.33 ± 0.58	4.33 ± 0.58	4.67 ± 0.58	5.67 ± 0.58	5.67 ± 0.58	6.33 ± 1.15	5.67 ± 1.15	5.00 ± 1.00	4.00 ± 1.00	4.67 ± 0.58	2.33 ± 1.73	1.33 ± 0.00	3.33 ± 1.15	4.67 ± 0.58	3.00 ± 1.00	2.67 ± 0.58	2.67 ± 0.58	1.00 ± 0.00	3.00 ± 1.00
Y	*0.07 ± 0.01*	0.18 ± 0.02	0.20 ± 0.04	0.21 ± 0.02	0.20 ± 0.01	0.11 ± 0.01	0.16 ± 0.02	0.17 ± 0.03	0.16 ± 0.03	0.26 ± 0.03	0.58 ± 0.13	0.14 ± 0.01	0.15 ± 0.03	0.12 ± 0.02	0.15 ± 0.03	0.13 ± 0.03	0.20 ± 0.02	0.19 ± 0.02	0.27 ± 0.04	0.15 ± 0.01
Zn	*8.53 ± 0.77*	15.17 ± 0.75	39.83 ± 7.18	27.77 ± 1.22	17.57 ± 1.55	10.73 ± 1.31	15.43 ± 0.12	12.50 ± 0.56	13.63 ± 1.65	19.20 ± 0.40	15.53 ± 1.50	15.27 ± 0.50	15.30 ± 4.50	13.33 ± 1.19	18.73 ± 3.09	22.07 ± 1.86	18.17 ± 0.68	50.73 ± 31.19	24.97 ± 3.89	26.57 ± 11.50
Zr	*0.21 ± 0.02*	0.68 ± 0.14	0.87 ± 0.18	1.07 ± 0.06	0.86 ± 0.05	0.32 ± 0.03	0.54 ± 0.04	0.48 ± 0.03	0.60 ± 0.04	1.02 ± 0.05	1.34 ± 0.10	0.50 ± 0.02	0.58 ± 0.11	0.41 ± 0.02	0.53 ± 0.06	0.69 ± 0.08	0.84 ± 0.05	0.75 ± 0.19	1.14 ± 0.15	0.59 ± 0.04

Table S1. Continued.

	MA2S	MU1G	MU1S	MU2G	MU2S	AcA1G	AcA1S	AcA2G	AcA2S	AcU1G	AcU1S	AcU2G	AcU2S	CA1G	CA2G	CA2S	CU1G	CU1S	CU2G	CU2S
Al	567 ± 58	367 ± 58	433 ± 58	400 ± 100	367 ± 115	667 ± 58	833 ± 252	367 ± 58	433 ± 58	300 ± 100	433 ± 58	667 ± 153	367 ± 58	533 ± 58	567 ± 58	467 ± 58	333 ± 58	467 ± 58	333 ± 58	300 ± 0
As	0.15 ± 0.12	0.05 ± 0.00	0.05 ± 0.00	0.20 ± 0.23	0.13 ± 0.12	0.07 ± 0.00	0.05 ± 0.00	0.10 ± 0.06	0.05 ± 0.00	0.05 ± 0.00	0.25 ± 0.15	0.23 ± 0.29	0.05 ± 0.00	0.25 ± 0.21	0.38 ± 0.36	0.47 ± 0.25	0.17 ± 0.17	0.18 ± 0.10	0.25 ± 0.21	0.40 ± 0.26
B	4.33 ± 0.58	2.67 ± 0.58	3.00 ± 0.00	2.67 ± 0.58	3.33 ± 0.58	4.33 ± 0.58	4.67 ± 0.58	4.33 ± 0.58	3.67 ± 0.58	3.33 ± 0.58	4.00 ± 0.00	5.00 ± 0.00	4.67 ± 0.58	6.00 ± 0.00	7.00 ± 1.00	6.67 ± 1.53	5.33 ± 0.58	5.67 ± 0.58	6.00 ± 1.00	4.00 ± 0.00
Ba	16.67 ± 0.67	15.77 ± 1.05	17.67 ± 0.55	15.57 ± 0.92	22.30 ± 11.79	18.93 ± 0.64	20.27 ± 0.40	15.60 ± 0.44	14.93 ± 0.50	15.40 ± 0.26	18.07 ± 0.68	19.87 ± 0.95	16.43 ± 1.25	17.10 ± 0.36	16.47 ± 0.38	17.50 ± 2.50	15.63 ± 1.60	18.33 ± 0.25	15.13 ± 0.57	14.30 ± 0.20
Ca	5167 ± 252	4500 ± 100	5833 ± 153	5000 ± 300	5533 ± 289	5333 ± 58	5767 ± 153	5033 ± 58	4933 ± 58	4467 ± 115	5767 ± 208	7533 ± 153	5067 ± 58	5233 ± 252	5033 ± 321	5300 ± 500	4667 ± 252	6067 ± 351	4767 ± 58	4933 ± 208
Cd	0.08 ± 0.01	0.08 ± 0.02	0.07 ± 0.02	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.09 ± 0.03	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.09 ± 0.02	0.07 ± 0.01	0.08 ± 0.02	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.02
Ce	0.80 ± 0.04	0.42 ± 0.02	0.65 ± 0.05	0.48 ± 0.16	0.54 ± 0.04	1.08 ± 0.02	1.29 ± 0.04	0.58 ± 0.04	0.49 ± 0.03	0.41 ± 0.02	0.74 ± 0.06	1.09 ± 0.09	0.54 ± 0.03	0.89 ± 0.04	0.68 ± 0.04	0.87 ± 0.14	0.42 ± 0.04	0.64 ± 0.06	0.42 ± 0.03	0.42 ± 0.03
Co	0.19 ± 0.03	0.10 ± 0.03	0.18 ± 0.02	0.12 ± 0.02	0.18 ± 0.04	0.21 ± 0.03	0.24 ± 0.01	0.15 ± 0.02	0.13 ± 0.02	0.11 ± 0.03	0.15 ± 0.04	0.25 ± 0.04	0.10 ± 0.01	0.17 ± 0.04	0.16 ± 0.01	0.17 ± 0.07	0.14 ± 0.03	0.15 ± 0.03	0.12 ± 0.01	0.15 ± 0.06
Cr	2.70 ± 0.26	2.93 ± 0.32	3.40 ± 0.26	2.93 ± 0.42	3.03 ± 0.06	3.20 ± 0.26	3.23 ± 0.15	2.87 ± 0.29	2.83 ± 0.15	2.70 ± 0.10	2.97 ± 0.15	3.13 ± 0.29	2.73 ± 0.15	2.83 ± 0.21	2.97 ± 0.21	2.83 ± 0.21	2.80 ± 0.30	3.07 ± 0.15	2.67 ± 0.12	2.63 ± 0.21
Cs	0.13 ± 0.00	0.08 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.00	0.18 ± 0.01	0.21 ± 0.00	0.10 ± 0.00	0.09 ± 0.00	0.08 ± 0.01	0.12 ± 0.01	0.17 ± 0.02	0.10 ± 0.01	0.14 ± 0.00	0.11 ± 0.00	0.13 ± 0.02	0.08 ± 0.01	0.11 ± 0.01	0.08 ± 0.01	0.08 ± 0.00
Cu	7.14 ± 0.61	4.56 ± 0.58	7.33 ± 0.56	4.25 ± 0.23	4.48 ± 0.73	8.71 ± 1.05	10.38 ± 0.30	6.47 ± 0.09	5.47 ± 0.24	5.29 ± 0.05	7.87 ± 0.41	9.20 ± 0.34	6.18 ± 0.14	6.69 ± 0.35	5.88 ± 0.29	6.03 ± 0.58	5.27 ± 0.27	7.36 ± 0.52	4.58 ± 0.19	4.91 ± 0.04
Fe	563 ± 6	440 ± 27	593 ± 70	477 ± 86	510 ± 46	687 ± 21	720 ± 20	493 ± 6	473 ± 6	427 ± 6	563 ± 32	740 ± 60	480 ± 10	603 ± 31	563 ± 23	600 ± 85	467 ± 25	580 ± 17	450 ± 20	430 ± 30
Hf	0.03 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.01
Hg	51 ± 20	38 ± 7	47 ± 9	52 ± 12	50 ± 1	49 ± 6	50 ± 7	39 ± 3	46 ± 12	45 ± 8	45 ± 10	53 ± 3	49 ± 12	55 ± 8	71 ± 6	64 ± 7	61 ± 2	64 ± 8	50 ± 7	43 ± 4
K	2100 ± 100	1833 ± 115	1833 ± 153	1733 ± 58	1900 ± 265	2333 ± 115	1833 ± 58	2100 ± 100	2233 ± 58	2300 ± 0	2067 ± 58	1833 ± 58	2533 ± 58	2200 ± 0	2300 ± 0	2133 ± 58	2400 ± 100	2333 ± 231	2133 ± 153	2133 ± 58
La	0.40 ± 0.01	0.21 ± 0.03	0.30 ± 0.02	0.24 ± 0.05	0.23 ± 0.04	0.50 ± 0.03	0.64 ± 0.02	0.26 ± 0.02	0.24 ± 0.01	0.19 ± 0.02	0.35 ± 0.04	0.54 ± 0.05	0.24 ± 0.03	0.54 ± 0.18	0.31 ± 0.02	0.42 ± 0.10	0.21 ± 0.03	0.33 ± 0.03	0.20 ± 0.03	0.20 ± 0.01
Li	0.36 ± 0.03	0.24 ± 0.06	0.28 ± 0.03	0.24 ± 0.07	0.31 ± 0.04	0.43 ± 0.02	0.46 ± 0.05	0.30 ± 0.03	0.26 ± 0.02	0.22 ± 0.06	0.26 ± 0.03	0.41 ± 0.03	0.29 ± 0.04	0.38 ± 0.06	0.37 ± 0.02	0.42 ± 0.07	0.23 ± 0.04	0.32 ± 0.07	0.23 ± 0.02	0.20 ± 0.02
Mg	960 ± 17	930 ± 17	990 ± 26	930 ± 44	927 ± 45	1037 ± 15	1090 ± 20	1017 ± 12	937 ± 15	897 ± 15	967 ± 6	1103 ± 38	920 ± 10	1093 ± 21	1160 ± 108	1137 ± 135	993 ± 57	1060 ± 26	957 ± 25	897 ± 21
Mn	16 ± 1	11 ± 1	15 ± 1	11 ± 2	12 ± 1	18 ± 1	21 ± 0	14 ± 1	13 ± 1	12 ± 0	14 ± 1	20 ± 1	14 ± 1	19 ± 1	15 ± 1	19 ± 2	13 ± 1	16 ± 1	13 ± 1	35 ± 17
Mo	0.31 ± 0.02	0.25 ± 0.02	0.36 ± 0.02	0.25 ± 0.04	0.24 ± 0.03	0.32 ± 0.02	0.33 ± 0.04	0.23 ± 0.03	0.24 ± 0.01	0.24 ± 0.02	0.31 ± 0.04	0.34 ± 0.03	0.30 ± 0.03	0.35 ± 0.02	0.29 ± 0.05	0.34 ± 0.03	0.25 ± 0.02	0.35 ± 0.01	0.25 ± 0.02	0.22 ± 0.03
Na	1563 ± 67	1237 ± 51	1340 ± 89	1187 ± 49	1270 ± 223	1760 ± 147	1603 ± 163	1603 ± 45	1477 ± 31	1367 ± 15	1480 ± 30	1430 ± 17	1673 ± 32	1800 ± 10	1947 ± 126	1857 ± 110	1537 ± 59	1623 ± 65	1377 ± 90	1360 ± 30
Nb	0.14 ± 0.02	0.04 ± 0.00	0.08 ± 0.02	0.05 ± 0.02	0.07 ± 0.01	0.21 ± 0.00	0.22 ± 0.03	0.07 ± 0.01	0.05 ± 0.01	0.04 ± 0.00	0.11 ± 0.01	0.18 ± 0.01	0.07 ± 0.01	0.15 ± 0.02	0.07 ± 0.01	0.13 ± 0.04	0.05 ± 0.01	0.07 ± 0.00	0.03 ± 0.01	0.04 ± 0.01
Ni	1.67 ± 0.85	1.03 ± 0.15	1.13 ± 0.06	0.97 ± 0.15	1.10 ± 0.10	1.23 ± 0.15	1.20 ± 0.10	1.17 ± 0.06	0.90 ± 0.00	0.93 ± 0.15	1.00 ± 0.10	1.17 ± 0.12	1.10 ± 0.10	1.20 ± 0.10	1.17 ± 0.15	1.10 ± 0.10	1.30 ± 0.10	1.17 ± 0.06	1.07 ± 0.12	0.93 ± 0.06
P	863 ± 25	777 ± 21	793 ± 47	720 ± 20	813 ± 32	887 ± 21	830 ± 17	837 ± 32	877 ± 15	913 ± 15	847 ± 15	877 ± 45	983 ± 32	973 ± 32	943 ± 45	930 ± 46	967 ± 38	950 ± 30	873 ± 31	840 ± 44
Pb	2.86 ± 0.17	2.13 ± 0.46	3.46 ± 0.11	1.38 ± 0.18	1.92 ± 0.31	2.66 ± 0.11	3.72 ± 0.08	1.52 ± 0.02	1.24 ± 0.05	1.19 ± 0.02	1.84 ± 0.08	3.48 ± 0.16	2.32 ± 0.51	3.45 ± 0.23	2.07 ± 0.12	2.32 ± 0.50	1.59 ± 0.18	1.95 ± 0.30	2.13 ± 0.87	1.61 ± 0.34
S	700 ± 557	600 ± 200	800 ± 346	467 ± 115	1167 ± 404	833 ± 252	833 ± 351	1033 ± 153	967 ± 321	1067 ± 321	183 ± 100	550 ± 503	867 ± 153	1033 ± 231	2000 ± 1179	2033 ± 961	2567 ± 252	2467 ± 751	1500 ± 529	517 ± 379
Sb	0.74 ± 0.08	0.74 ± 0.10	0.98 ± 0.27	0.67 ± 0.19	0.77 ± 0.25	1.22 ± 0.21	1.02 ± 0.05	0.68 ± 0.06	0.93 ± 0.06	0.64 ± 0.03	0.91 ± 0.06	0.78 ± 0.04	0.83 ± 0.07	0.85 ± 0.09	1.01 ± 0.16	0.86 ± 0.10	1.14 ± 0.23	0.95 ± 0.24	0.59 ± 0.06	0.94 ± 0.12
Sc	0.47 ± 0.10	0.37 ± 0.06	0.40 ± 0.05	0.40 ± 0.08	0.37 ± 0.08	0.30 ± 0.05	0.40 ± 0.05	0.37 ± 0.06	0.30 ± 0.08	0.37 ± 0.06	0.37 ± 0.06	0.33 ± 0.06	0.33 ± 0.10	0.30 ± 0.05	0.33 ± 0.06	0.40 ± 0.05	0.47 ± 0.06	0.33 ± 0.06	0.37 ± 0.06	0.33 ± 0.08
Se	0.20 ± 0.00	0.33 ± 0.15	0.40 ± 0.10	0.18 ± 0.10	0.37 ± 0.12	0.43 ± 0.12	0.25 ± 0.15	0.40 ± 0.10	0.37 ± 0.12	0.30 ± 0.10	0.50 ± 0.10	0.40 ± 0.10	0.27 ± 0.12	0.30 ± 0.10	0.33 ± 0.21	0.37 ± 0.15	0.27 ± 0.12	0.47 ± 0.12	0.15 ± 0.12	0.23 ± 0.06
Sn	0.44 ± 0.06	0.41 ± 0.09	0.67 ± 0.09	0.34 ± 0.05	0.41 ± 0.06	0.48 ± 0.03	0.56 ± 0.08	0.33 ± 0.07	0.28 ± 0.04	0.31 ± 0.04	0.56 ± 0.04	0.70 ± 0.04	0.42 ± 0.07	0.38 ± 0.06	0.34 ± 0.04	0.38 ± 0.08	0.29 ± 0.05	0.52 ± 0.05	0.27 ± 0.03	0.26 ± 0.04
Sr	11.23 ± 0.32	10.10 ± 0.26	11.47 ± 0.42	10.50 ± 0.61	11.03 ± 1.40	12.40 ± 0.17	13.77 ± 0.23	11.20 ± 0.20	10.67 ± 0.25	9.57 ± 0.12	11.53 ± 0.38	14.73 ± 0.31	10.57 ± 0.12	12.47 ± 0.49	12.47 ± 0.91	12.77 ± 1.75	10.53 ± 0.64	12.30 ± 0.20	10.27 ± 0.40	10.13 ± 0.21
Th	0.09 ± 0.00	0.04 ± 0.00	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.00	0.11 ± 0.00	0.12 ± 0.01	0.07 ± 0.00	0.05 ± 0.01	0.04 ± 0.01	0.08 ± 0.01	0.10 ± 0.01	0.06 ± 0.01	0.09 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.05 ± 0.00	0.07 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
Ti	16.00 ± 0.00	8.67 ± 0.58	12.00 ± 1.00	9.33 ± 1.53	10.67 ± 0.58	21.67 ± 0.58	26.00 ± 0.00	12.00 ± 0.00	10.67 ± 0.58	8.67 ± 0.58	14.00 ± 0.00	21.00 ± 2.65	11.00 ± 0.00	15.33 ± 1.15	11.67 ± 0.58	15.33 ± 2.08	9.33 ± 0.58	12.33 ± 0.58	8.33 ± 0.58	8.00 ± 0.00
Tl	0.03 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	0.02 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.01
U	0.05 ± 0.01	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.03 ± 0.00	0.03 ± 0.01	0.02 ± 0.01	0.04 ± 0.01	0.06 ± 0.01	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.00	0.04 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.00
V	3.00 ± 0.00	4.33 ± 1.15	5.67 ± 1.53	4.67 ± 0.58	6.00 ± 1.73	4.67 ± 1.15	5.33 ± 0.58	5.00 ± 1.00	3.33 ± 1.53	1.00 ± 0.00	1.67 ± 0.00	2.33 ± 0.58	2.33 ± 0.58	3.33 ± 0.58	5.67 ± 0.58	4.67 ± 0.58	5.00 ± 0.00	4.00 ± 1.00	3.00 ± 1.00	2.00 ± 0.58
Y	0.19 ± 0.02	0.10 ± 0.01	0.18 ± 0.03	0.12 ± 0.01	0.14 ± 0.01	0.25 ± 0.01	0.29 ± 0.04	0.14 ± 0.00	0.13 ± 0.01	0.12 ± 0.00	0.17 ± 0.01	0.25 ± 0.03	0.13 ± 0.01	0.21 ± 0.02	0.17 ± 0.02	0.21 ± 0.04	0.11 ± 0.01	0.17 ± 0.03	0.12 ± 0.01	0.11 ± 0.00
Zn	13.27 ± 1.21	11.77 ± 0.49	15.47 ± 0.59	11.87 ± 0.49	117.87 ± 177.66	17.93 ± 0.21	21.13 ± 0.65	83.63 ± 5.52	10.80 ± 0.56	10.07 ± 0.55	14.87 ± 0.75	22.50 ± 1.06	18.10 ± 0.56	13.20 ± 0.62	103.90 ± 48.02	192.93 ± 35.62	9.97 ± 1.46	116.97 ± 15.37	9.70 ± 0.10	9.77 ± 0.21
Zr	0.90 ± 0.06	0.35 ± 0.03	0.63 ± 0.07	0.40 ± 0.04	0.48 ± 0.03	0.89 ± 0.03	1.06 ± 0.10	0.50 ± 0.02	0.41 ± 0.04	0.31 ± 0.02	0.67 ± 0.02	0.89 ± 0.03	0.47 ± 0.02	0.81 ± 0.09	0.54 ± 0.03	0.77 ± 0.11	0.33 ± 0.04	0.64 ± 0.11	0.33 ± 0.02	0.35 ± 0.07

Chapter 3

Air pollution monitoring using emission inventories combined with the moss bag approach

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Abstract

Inventory of emission sources and biomonitoring with moss transplants are two different methods to evaluate air pollution. In this study, for the first time, both these approaches were simultaneously applied in five municipalities in Campania (southern Italy), deserving attention for health-oriented interventions as part of a National Interest Priority Site. The pollutants covered by the inventory were CO, NO_x, particulate matter (PM_{10}), volatile organic compounds (VOCs), and some heavy metals (As, Cd, Cr, Cu, Hg, Ni, Pb, Se, and Zn). The biomonitoring survey was based on the use of the devitalized moss *Hypnum cupressiforme* transplanted into bags, following a harmonized protocol. The exposure covered 40 agricultural and urban/residential sites, with half of them located in proximity to roads. The pollutants monitored were Al, As, Cd, Cr, Cu, Fe, Hg, Ni, Pb, Se, and Zn, as well as total polycyclic aromatic hydrocarbons (PAHs) only in five sites. Using the emission inventory approach, high emission loads were detected for all the major air pollutants and the following heavy metals: Cr, Cu, Ni, Pb and Zn, over the entire study area. Arsenic, Pb, and Zn were the elements most accumulated by moss. Total PAH postexposure contents were higher than the preexposure values (~20–50% of initial value). Moss uptakes did not differ substantially among municipalities or within exposure sites. In the five municipalities, a similar spatial pattern was evidenced for Pb by emission inventory and moss accumulation. Both approaches indicated the same most polluted municipality, suggesting their combined use as a valuable resource to reveal contaminants that are not routinely monitored.

Keywords: *Air quality, pollution sources, emission scenarios, road transport, biomonitoring, Hypnum cupressiforme, heavy metals, PAHs*

Highlights

- Emission inventory and moss biomonitoring were at once used to assess air pollution.
- The road transport contributed most of the emissions of CO, NOx, PM_{10}, Cu, Pb, and Zn.
- Emission inventory and moss accumulation evidenced similar spatial patterns for Pb.
- The two approaches indicated the same most polluted municipality.
- Both approaches monitored point and diffuse source pollution.

1. Introduction

The inventory of emission sources is a primary cognitive method both to examine the origin of air pollution and to evaluate the air quality state in areas subject to anthropogenic activities. Such emission inventories entail collection of technological, geographical, and social data for urban planning and air quality management, and contribute to detect the different air pollution sources with spatial disaggregation (regional, provincial, and urban) and evaluate qualitatively and quantitatively the air pollutants emitted from different sources, such as industries, transportation, and housing (Iodice and Senatore, 2013a).

Emission inventories are essential tools to recognize the zones where noncompliance with limit standards may occur, because they can provide direct and current information on the emissions load in critical areas. They are chief cognitive instruments to organize the input information for atmospheric dispersion models that, in compliance to the EU Air Quality Directive, can be used to assess air quality state under well-itemized environmental situations (EEA, 1998).

Biomonitoring using moss transplants is an alternative way to evaluate air pollution. This method is widely used in Europe, and has been recently reviewed by Ares et al. (2012). The use of mosses has several advantages over the current physicochemical methods, such as simultaneous monitoring of a large number of contaminants (i.e., metals and metalloids, polycyclic aromatic hydrocarbons (PAHs), and radionuclides), easy application, cost-effectiveness with dense sampling networks, and no need of energy supply. Moreover, there is a lack of studies devoted to validate moss transplants over the currently used conventional methods for air pollution monitoring and comparison with dispersion models based on inventories of emission sources (Adamo et al., 2008; De Nicola et al., 2013).

In recent years, Campania (southern Italy), one of the most fertile agricultural landscapes in Italy, has become the focus of attention of the media, because its soil has been legally and illegally used to discharge potentially toxic waste. Moreover, in time, the problem has been aggravated by people setting fire to illegal waste disposal sites. It is widely held that this situation is the reason for the increase in the incidence of cancer and the shorter life expectancy of people living in the areas affected (Senior and Mazza, 2004). In pilot sites of "Litorale Domizio-Agro Aversano," recognized by the Italian State as a Regional Interest Priority Site (RIPS) in Campania, the Life ENV-IT 275 ECOREMED (www.ecoremed.it) project is ongoing to implement ecologically compatible protocols for agricultural soil remediation. In this framework, this study aims to evaluate the air pollution of five municipalities located in this RIPS, combining data from the emission inventories of pollutants and a biomonitoring survey. The emission inventories were based on direct measurements, only for the main industrial systems (point sources), and these measurements are the emission levels detected on the stacks of point sources, provided by the same industries. The pertinent measured substances are the principal air pollutants and, for some industrial systems, heavy metals. On the contrary, for the main nonpoint sources, the emission inventories were based on indicators and factors of emissions activity. The pollutants covered by the inventory presented in this study are carbon monoxide (CO), nitrogen oxides (NO_x), particulate matter (PM_{10}), volatile organic compounds (VOCs), and some heavy metals (As, Cd, Cr, Cu, Hg, Ni, Pb, Se, and Zn).

A biomonitoring survey based on the use of moss bags was conducted simultaneously in the same five towns to assess the uptake of heavy metals and PAHs. In addition, a comparison was made between the data obtained from biomonitoring survey and estimated emissions, with special attention to the road transport sector, which is still a major source of air pollution despite extensive action worldwide to abate such emissions (Mellios et al., 2006).

2. Materials and Methods

2.1 The study area

Litorale Domizio-Agro Aversano in Campania, with a total area of approximately 200,000 ha, encompasses the river plains of the Garigliano and Volturno and partly the Phlegrean Fields. It includes much of the agricultural land belonging to more than 61 municipalities in the provinces of Naples and Caserta (Bove et al., 2011). In the area under investigation, intensive agriculture is mainly characterized by fodder crops (mostly maize and alfalfa), vegetable crops, orchards, and buffalo livestock. Production in the area of the famous buffalo mozzarella cheese was recognized by

Ministerial Decree in May 1993 and awarded the Protected Denomination of Origin (PDO) certification (European communities 1992; 1996). Unfortunately, the area has also experienced continuous, widespread land degradation mainly because of chaotic urbanization. Agricultural practices, the presence of numerous landfill sites (both legal and illegal), the usual practice of waste incineration, and leakage from the sewage network have resulted in soil and groundwater contamination, with water in many wells exhibiting very high nitrate concentrations and point source pollution (heavy metals, hydrocarbons, and pesticides) (Capra et al., 2014; Corniello and Ducci, 2014; Grezzi et al., 2011). In addition, it was formerly identified by the Italian State as one of the 57 National Interest Priority Sites (NIPS, L. 426/98), where severe environmental pollution and degradation are predicted to occur. Since January 2014, it has been recognized as a RIPS (D.M. 11 January 2013), assigning any land restoration activity to the Campania Regional Authority. Within this area, five towns, namely Acerra, Casal di Principe, Giugliano, Maddaloni, and Teverola, were chosen for emission inventory and biomonitoring data comparison (Fig. 1), whose populations were 59,578; 21,351; 120,194; 39,196; and 13,346, respectively.

2.2 Emission Inventory

The atmospheric emission inventories can be enucleated as a broad set of data connected to emissions of air pollutants from natural and anthropogenic activities. Such information must comprise the chemical characteristics of the air pollutants, quantifying the natural and human activities accountable for emissions, the emission factors or the data required for their estimation, and the position and time variation of the emissions for all activities. At the European level, the atmospheric emission inventories are drawn up in compliance to CORINAIR methodology, as described in detail in Ohara et al. (2007).

In this study, the emissions of the examined pollutants were assessed following CORINAIR methodology for four anthropogenic activity sectors: transformation/power (electricity and heat generation, petroleum refineries, and similar energy and transformation industries); industry (iron and steel, chemical and petrochemical, and nonferrous plants); road transport, and others including agriculture, commerce, and residential (Iodice and Senatore, 2015a). The amounts of pollutants emitted from the several emissive sources in the five towns under examination were calculated by current and real measures where conceivable, or else by estimations.

The pollutant emissions were directly measured only for the main industrial systems, that is, large industrial sources that are ruled by licenses and regulations or are demanded to report their emission levels because of their dimension or productive capacity. More specifically, emissions were measured only for those plants (point sources) characterized by emissive levels >50 Mg/year for CO, >50 kg/year for heavy metals, and >5 Mg/year for all the other air pollutants. For all other sources (then also small facilities), the emission levels were estimated as produced by nonpoint sources. In order to obtain direct measurements for the relevant point sources (i.e., the chief stack sets of longitudinal and latitudinal spatial coordinates for each examined chimney), surveys were conducted in the main factories in the towns concerned, which subsequently measure the emission levels of the principal air pollutants (CO, NO_x, VOCs, and PM_{10}) and, for some industrial systems, also heavy metals. Within the five municipalities, these point sources particularly comprised power plants and industrial boilers running on different fuels such as natural gas, liquefied petroleum gas, diesel, and coal. The pertinent emissive levels were first estimated for every point source and, in the next phase, for each air pollutant, all the emissions from punctual sources situated in the same town were summed.

For all other emission sources, namely nonpoint sources (urban road transport, small enterprises, natural sources, heating systems), emissions were appraised by collecting data related to activity data, emission factors, and removal effectiveness of emission controls, as shown by Ohara et al. (2007), gathering (mostly statistical) information from agencies and institutions or from the literature. Region-detailed emission factors were collected from an extensive literature, such as AP-42 (U.S. EPA, 1999), the Intergovernmental Panel on Climate Change Guidelines (IPCC, 1997), and the EMEP/CORINAIR Emission Inventory Guidebook (EEA, 2009). The obtained emission factors were then used to assess emissions for each pollutant at a municipality level, considering data available for the year 2012.

2.3 Emissions from the road transport sector: uncertainties and calculation procedure

Despite their great significance, emissions from road traffic sector are more complex to estimate than other anthropogenic activities, as they depend on several variables that are subject to a fair degree of uncertainty. Such uncertainties are both intrinsic in emission factors (that are dependent on the air pollutant, vehicle and fuel type, and service and maintenance programs) and related to the numerous factors essential for their assessment (driving patterns, fuel consumptions, annual mileages, and climatic aspects).

In this study, air emissions resulting from the road traffic sector were assessed by a method based on the COPERT methodology (Gkatzoflias et al., 2012), that is, a database of algorithms and emission factors proposed at European level for the estimation of road transport emissions (CORINAIR 1988, Ntziachristos and Samaras 2012). On the basis of this methodology, the estimate of the air emissions is possible for 230 vehicle categories grouped into five main classes: passenger cars, light-duty vehicles, heavy-duty vehicles, urban buses and coaches, and two wheelers (mopeds and motorcycles), further subdivided depending on fuel type, the EU directives for their emission limits, and the engine displacement. Emissions can be calculated for 36 pollutants, including PAHs, dioxins, and heavy metals present in the fuel. The COPERT procedure can be used with a top-down or bottom-up approach depending on the resolution of the data set. In this study, the COPERT procedure is used with the bottom-up approach, then focusing on the municipal level in lieu of national level, thus comprising local and certain variables concerning driving patterns, the vehicle fleet, average trips, average speeds, and fuel consumptions. The selection and gathering of these basic input data to compute the emissions from road transport involved the following organizations and societies:

- the Italian Institute of Statistics (ISTAT);
- the Ministry of Transport and Navigation (MIT, 2011);
- the Italian Institute for Environmental Protection and Research (ISPRA, 2010);
- the Italian Automotive Association (ACI), which provides the number of registered vehicles and their composition at municipality level (ACI, 2011);
- the Italian Association of Oil Companies, which provides the fuel sold for each Italian province in its annual oil market bulletins (MICA, 2011).

The base year for the appraisal of this emission inventory is 2012.

2.4 Biomonitoring survey

2.4.1 Experimental design of moss bag exposure

The exposure methodology was used following a harmonized protocol (Ares et al., 2012), in which devitalized moss *Hypnum cupressiforme* Hedw. was transplanted into subspherical bags exposed for 6 weeks (March–April 2014) in the five towns of Campania under study. The exposure design was set by considering the inventory of known atmospheric emissions and the diffuse urbanization of the area, in which agricultural and urban/residential sites are strictly intermingled. In order to highlight the effects of vehicle traffic on moss uptake, in each municipality, eight different exposure points were chosen, four of which were close to main roads, summing to 40 exposure points.

For the preparation of bags, moss *H. cupressiforme* was collected in the natural site of Gabrovizza (Sgonico, Northeast Italy), which was considered as "clean" area, and processed according to Ares et al. (2012). Briefly, moss was manually cleaned after collection, green shoots were selected and washed in distilled water and ethylenediaminetetraacetic acid (EDTA), and devitalized in an oven at 120°C for 24 h. Unexposed *H. cupressiforme* was considered as baseline moss (T0) and stored at room temperature (20°C) and low temperature (4°C) for metal and PAH analyses, respectively. About 500 mg of moss was taken in subspherical bags prepared with a nylon net (2 mm mesh), suspended on latticework by nylon strings, and exposed 4 m above ground level, in triplicate for metal and PAH analyses. Because of analytical constraints (shortage of naturally growing *H. cupressiforme* and budget limitations), it was decided to expose moss only in five sites (one for each municipality) principally to test the validity of our method for PAH assay, as the use of cryptogams and related analytical methods to evaluate PAHs is still in the beginning stage.

2.4.2 Analytical procedures

Metals and metalloids

After exposure, the moss material removed from each nylon bag was oven-dried at 40°C, processed and analyzed separately. The material was first milled and homogenized using a Retsch PM200 ball mill equipped with agate pockets. For metal and metalloid analysis, pre- and postexposure moss samples were mineralized with ACS-grade HNO_3 for 1 h and then with *aqua regia* (ACS-grade HCl–HNO_3 with a volume ratio of 1:3) in a boiling water bath (95°C) for 1 h. Sample solutions were analyzed using a Perkin Elmer Elan 6000 inductively coupled plasma (ICP) mass spectrometer. All concentrations were reported on a dry weight basis. Procedural blanks were usually below detection limits and M3-certified reference moss from the Finnish Forest Research Institute (Steinnes et al., 1997) was used to check the accuracy and precision of the digestion and ICP analysis procedure. In total, 37 elements were analyzed. In this study, data for only the following elements are considered: Al, As, Cd, Cr, Cu, Fe, Hg, Ni, Pb, Se, and Zn. The percentage of recovery was >70% for all the elements except As, which resulted under limits of detection in the reference material. The relative standard deviations were <20%, except for Cr, Se, and Cd (42%, 33%, and 32%, respectively).

PAHs

The PAH concentrations in moss samples were determined after extraction (Sonicator XL2020, Misonix Inc., USA) in a dichloromethane–acetone mixture (1:1 v/v) through three sonications. The extracts were evaporated by a rotary vacuum evaporator, purified by polytetrafluoroethylene (PTFE) filters, and dried in a mild nitrogen stream. The samples were then resuspended with cyclohexane and analyzed by gas chromatography–mass spectrometry (GC–MS) (HP 5890–HP 5971 with HP-5MS capillary column 30 m × 0.25 mm (i.d.) × 0.25 μm (film thickness); Agilent Technologies Inc., USA). All analyses were performed in selected ion monitoring (SIM) mode. The concentrations of 20 PAHs were quantified by multipoint calibration curves and labeled internal standards. For quality control of the procedure, labeled PAHs (naphthalene D8, acenaphthylene D8, fluoranthene D10, benzo(k)fluoranthene D12, and benzo(g,h,i)perylene D12) were used as surrogates and the percentage recoveries (70–130%) included correcting the concentration of each compound. The minimum detectable PAH concentration was 0.001 mg kg^{-1} (dry weight) for each compound.

2.4.3 Data analyses

Metal and PAH concentration data were processed by basic statistics using Microsoft Excel and by principal component analysis (PCA) using Statistica StatSoft 6.0. The element concentrations below the limit of detection (LOD) were assumed as LOD/2. The formula proposed by Frati et al. (2005) was used to evaluate whether moss transplants accumulated elements during exposure. This formula considers the ratio of the concentration of each element after exposure to that of the T0 sample before exposure (exposed-to-T0 ratio, EC ratio), producing an interpretative scale of five classes (Table 1) of accumulation/loss based on progressive ±25% deviations from "normal" conditions, assumed to be ±25% from the ratio of 1.

3. Results and Discussion

3.1 Emission inventory

Overall emissions assessed in the five municipalities examined for the road transport sector and for all the SNAP sectors are summarized in Table 2, which provides the sum of the major air pollutants (CO, NO_x, PM_{10}, and VOCs) and some heavy metals produced from nonpoint and point sources, presented in tons (Mg) and kilograms (kg) per year. It is evident from the table that the amounts of all these emissions are also unbundled for CORINAIR sectors of activity.

Because the total area of the five municipalities is approximately 216 km^2, the emission load in the areas concerned, resulting from this investigation, is consistent for all the major air pollutants and the following heavy metals: Cr, Cu, Ni, Pb and Zn, caused by the anthropogenic activities affecting the areas under examination.

In order to achieve a comparison of the emission load among the five municipalities, characterized by different values of surface areas, total emissions of all air pollutants under study were broken down by municipality and divided by the relevant surface area, thus obtaining the specific emissions shown in Table 3. In this study, the dispersion of atmospheric pollutants with the use of mathematical models has not been analyzed, but an implicit relationship between the specific emissions so obtained and the pollutant dispersions has been assumed, thereby hypothesizing indirectly a homogeneous and spatial equal atmospheric distribution of emissions. Analyzing the results of this emission inventory, Maddaloni and Teverola represent the towns with the greatest annual emission load for all the investigated pollutants; however, the lowest emission load was detected in Casal di Principe.

In order to determine the possible causes of this emission scenario and to justify the differences in the emission levels among the five municipalities, the total annual emissions of the major air pollutants and Cu, Pb, and Zn are provided in Figures 2 and 3, indicating the contribution of the road transport sector for each municipality.

In Giugliano and Casal di Principe, the road transport sector is the predominant contributor of the emission levels of CO, NO_x, PM_{10}, Cu, Pb, and Zn, whereas in Acerra, Maddaloni, and Teverola, the contribution of industrial production processes and combustion in manufacturing industries (burning oil, coal or natural gas) is as high as that of the road transport sector.

In the five municipalities, CO emissions are mainly due to the road transport sector (73%), followed by combustion in manufacturing industries burning oil or natural gas (12%). Industrial oil and natural gas use is the major emission sector for NO_x (49%), followed by road transport and industrial production processes (16% and 12%, respectively). For PM_{10} emissions, road transport and nonroad traffic sectors contribute 35% and 16% respectively, industrial sources (burning natural gas, oil, or coal) account for approximately 17%, and the percentage of natural gas and LPG combustion in the residential sector is about 7%. In the studied area, as in the rest of southern Italy, particulate emissions are influenced by decreasing the contribution from oil and coal combustion in the residential sector and increasing the emissions from diesel cars (Gimelli et al., 2012; Iodice and Senatore, 2013b). For VOCs, road traffic, solvent and paint use, and production process sectors contribute 40%, 33%, and 10% to overall emissions, respectively.

These results reveal that for the studied municipalities (as for the whole of Campania), road traffic is a consistent contributor to total emissions. This finding can be correlated to the high average age of the car fleet and motorcycles in the region; the modernization of this fleet, in fact, is much slower than in other Italian regions, thereby causing unavoidable consequences on emission levels of these pollutants.

In particular, petrol-fueled cars contribute to about 46% of the total emissions of CO from the road traffic (Iodice and Senatore, 2014a), because of their higher mileage (27%) than other vehicle classes and also because these vehicles generally travel at low speed in urban contexts. Hence, the combustion quality is far from efficient, with a resulting increase in CO emissions. Similarly, motorcycles and mopeds contribute to 17% and 13%, respectively, of total CO emissions because of the incomplete combustion in petrol-powered engines.

The high emissions of CO and HC from road transport sector can be attributed to incomplete combustion in driving conditions with a steep rise in engine speed that is no longer offset by the catalytic converter. However, for new vehicles, belonging to the most recent EU emission classes, both internal engine optimization and a more precise mixture setting of fuel injection systems achieve a better control of fuel consumption, improving the efficiency of the catalytic converter.

Petrol-fueled cars are a major contributor of VOC emissions (~36%) (Iodice and Senatore, 2014a). A surprising outcome is the 28% of VOC emissions attributable to mopeds, despite their much lower average mileage. Indeed, Iodice and Senatore (2014b, 2015b) showed that many mopeds in the studied area are equipped with old-generation two-stroke engines and a carburetor devoid of an efficient electronic mixture control, thus producing a high VOC emission factor (~13 g/km). For this reason, the adoption and spread of four-stroke engines with direct injection in mopeds will help abate the CO and HC emissions.

3.2 Biomonitoring survey

The present data concern the nine investigated elements chosen according to the available inventory emission data together with Al and Fe as indicators of terrigenous contribution (Table 4) and PAHs (Table 7). Results in Table 4 indicate that in all the investigated municipalities, the content of most of the elements increased after 6 weeks of exposure and was almost homogeneous both among the five municipalities, and within each of them, as highlighted by the low coefficients of variation (in most cases <27%).

According to the EC ratios presented in Table 5, the moss exposed in 40 monitoring points showed a severe accumulation of As, Pb, and Zn; moderate accumulation of Al, Cr, Cu, Fe, and Ni; no accumulation of Cd and Hg; and loss of Se.

The correlations among the contents of the elements in samples are shown in Table 6. A highly significant positive correlation occurs between Al, Cr, Fe, and Pb, supporting the thesis of a terrigenous contribution to the accumulation of these elements by mosses.

PAH pre- and postexposure contents are reported in Table 7. Considering both total and only 2–4 ring PAHs, postexposure contents were always higher than the pre-exposure counterparts (from 19% to 53% of initial value). Postexposure contents are homogeneous among the replicates based on low standard deviation values, and no significant differences were observed among sites.

3.3 Comparison between the emission inventory and biomonitoring output

It is worth considering how available emission inventory data disaggregated for the five studied municipalities can be reconciled with the biomonitoring output. Considering this, we examined Cu, Pb, and Zn as heavy metals and VOC emissions versus 2–4 ring PAHs accumulating in moss. When comparing estimated emissions and bioaccumulation data, the former should be considered as annual-based (2011), and the latter provide information for a short exposure period (6 weeks in spring 2014).

3.3.1 Heavy metals

The total inventory emissions and the contribution from road transport for Cu, Pb, and Zn were compared with average moss accumulation in all exposure sites (n = 40) and only in road sites (n = 20), to check similarities among distribution patterns in each investigated municipality (Fig. 3).

In general, a similarity exists between total and road transport patterns both in emission inventory and biomonitoring survey graphs, indicating a key role of road transport in heavy metal air pollution. Considering only biomonitoring data, no substantial differences were observed in average moss accumulation of Cu, Pb, and Zn between all exposure sites and only road sites. Besides, moss accumulation data also suggest that road transport has a higher contribution toward Cu pollution in four municipalities and to Zn alone in Casal di Principe.

After ranking emission data and accumulation data, a very similar spatial trend between total emissions and moss accumulation in the five towns was observed only for Pb, whose average values were in the following order: emission inventory, Maddaloni > Teverola > Giugliano > Acerra > Casal di Principe; moss accumulation, Teverola > Maddaloni > Giugliano > Acerra > Casal di Principe. By contrast, different trends of the two data sets were observed for Cu and Zn, which showed similar qualitative information only in Acerra and Maddaloni.

The different time information considered in the two data sets may explain the discrepancies observed between the trends of Cu and Zn on the one hand and Pb on the other. Metal emissions can be variable in time and space, affecting in different ways the records for metals with single/multiple emission sources or with nonpoint, unrecorded sources. For example, the similar trend observed for Pb probably stems from the fact that road traffic is the main contributor of pollution levels. The accumulation/emission of lead in the study area can be directly related to the amount of fuel used by vehicles in the area. The trend found for Pb is not substantially variable in time and space, because its main source (vehicle traffic) is probably constant over time. The presence of Pb, as well as other heavy metals (e.g., Cd, Cu, Ni, and Zn), is mainly attributed to road traffic and vehicle emissions (U.S. EPA, 2001; Zhang et al., 2012). These elements can be deposited and resuspended in the form of dust (Al-Fatlawi and Al-Alwani, 2012), a complex mixture of particulates and contaminants deriving from various sources such as abrasion of bitumen, brakes, tires, and wheels (Pal et al., 2011; Sutherland et al., 2012).

The different trends observed for Cu and Zn may be due to the different and variable emission sources in the studied environment. Indeed, these two elements are widely used in agricultural practices and industrial processes (Kabata-Pendias, 2010). Moreover, metal accumulation by moss may also derive from additional sources, not considered by the emission inventory, such as the agricultural practices other than machinery (i.e., application of fertilizers and pesticides) and resuspension of polluted soil.

The total emissions recorded in the inventory (Mg/km^2) and the average accumulation in moss (mg/kg) from all the exposure sites in each municipality were analyzed and compared with PCA (Fig.

4). The data related to Cd, Hg, and Se were excluded from the analysis, because they were either not accumulated in moss samples or leached from moss tissue.

On the basis of the emission inventory, the five municipalities are mainly distributed along Factor 1, which explains about 50% of total variance, from the most affected Teverola to the least affected towns of Giugliano and Casal di Principe. Similarly, on the basis of biomonitoring data, Teverola appears to be the most affected municipality. The others seem to be affected differently by metal distribution along both factors. The differences highlighted by PCA analysis between emission and accumulation data could be explained by the different reference periods of the two data sets, as suggested earlier.

3.3.2 PAHs

Because the VOC data reported in the emission inventory include low-molecular weight PAHs, only the 2–4 ring PAHs were considered. The comparison between 2–4 ring PAH accumulation and VOC emissions is shown in Figure 5.

Biomonitoring outputs (Table 7) and total VOC emissions (Table 3) are not characterized by similar trends; the small number of sampling points does not enable a better comparison of the two data sets. As for heavy metals, the discrepancies observed between the two data sets could suggest different inputs, variable over space and time, which are absent or difficult to record in the usual inventories.

4. Conclusions

The uniqueness of this study lies in the combined use of the two widely used methods for evaluating air pollution: the inventory of emission sources and biomonitoring by moss transplants. Combined analysis of the different data sets clearly shows that for the investigated municipalities in Campania, a considerable part of total emissions is due to road traffic. When the emission inventory is based on a reliable and constant-over-time single pollution source, as in the case of lead, the two data sets produce almost coincident outputs, whereas those contaminants showing multiple and scattered sources, variable in time, produce less overlapping outputs.

Therefore, according to a harmonized protocol, the combined use of the atmospheric emission inventory and moss bags is an important tool to study air quality and set up possible remediation plans. This could be particularly useful in areas of alleged pollution, to support the monitoring of attainment of limit values established by legislation. In areas with high pollution, the creation of a spatially distributed emission inventory and the establishment of a regular biomonitoring network using moss bags could be part of a project to develop and improve the modeling of air quality. Indeed, the comparison between the two data sets (i.e., emission inventory and biomonitoring) could prove a valuable resource to reveal suspected point source pollution (where denser data points are needed) and for the simultaneous detection of contaminants not monitored by automatic devices and eluding the records of emission inventories. It is believed that this study will help engineers and research biologists to face the challenges of air pollution monitoring.

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Tables

Table 1. Interpretative scale of EC ratio

0-0,25	**severe loss**
0,25-0,75	**loss**
0,75-1,25	**normal**
1,25-1,75	**accumulation**
>1,75	**severe accumulation**

Table 2. Total annual emissions of main air pollutants (CO, NO_X, PM_{10} and VOCs) and some heavy metals over five Municipalities of Campania Region (Acerra, Casal di Principe, Giugliano, Maddaloni and Teverola), disaggregated for CORINAIR sectors.

CORINAIR sectors	**CO (Mg)**	**NO_X (Mg)**	**PM_{10} (Mg)**	**VOCs (Mg)**	**As (kg)**	**Cd (kg)**	**Cr (kg)**	**Cu (kg)**	**Hg (kg)**	**Ni (kg)**	**Pb (kg)**	**Se (kg)**	**Zn (kg)**
Combustion in energy and transformation industries	2.33	251	4.09	2.97	0.12	0.12	0.05	0.12	0.00	0.12	0.48	0.00	0.24
Nonindustrial combustion plants	119	74.5	31.4	12.1	1.15	0.72	1.16	0.72	0.24	15.7	1.15	0.02	3.79
Combustion in manufacturing industries	681	1945	78.9	89.3	4.99	5.68	20.2	6.68	3.09	199	13.4	0.04	10.7
Production processes	473	460	104	320	0.00	0.36	23.0	3.08	0.26	10.5	14.8	11.2	25.3
Extraction and distribution of fossil fuels/geoth. energy	0.00	0.00	0.00	37.9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Solvent and other product use	0.00	0.00	0.66	1060	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Road transport	4075	626	161	1270	0.00	0.41	2.04	69.5	0.00	2.86	32.8	0.41	40.9
Other mobile sources and machinery	196	632	75.1	93.6	0.00	0.45	2.25	76.4	0.00	3.15	28.5	0.45	44.9
Waste treatment and disposal	0.00	0.00	0.00	208	0.00	0.03	0.17	5.77	0.00	0.24	0.28	0.03	3.39
Agriculture (machineries)	6.25	0.12	0.65	75.2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Other sources	1.42	0.00	0.08	1.74	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Totals	**5550**	**3990**	**456**	**3170**	**6.26**	**7.77**	**48.9**	**162**	**3.59**	**231**	**91.5**	**12.2**	**129**

Table 3. Total annual emissions of main air pollutants and heavy metals in the examined area disaggregated for single Municipality (Acerra, Casal di Principe, Giugliano, Maddaloni and Teverola).

	CO Mg/m^2	**NO_X** Mg/m^2	**PM_{10}** Mg/m^2	**VOCs** Mg/m^2	**As** kg/m^2	**Cd** kg/m^2	**Cr** kg/m^2	**Cu** kg/m^2	**Hg** kg/m^2	**Ni** kg/m^2	**Pb** kg/m^2	**Se** kg/m^2	**Zn** kg/m^2
Acerra	21.9	27.8	1.74	17.3	0.08	0.08	0.22	0.69	0.03	2.86	0.35	0.00	0.44
Casal di Principe	18.5	4.78	1.20	9.71	0.00	0.01	0.02	0.58	0.00	0.11	0.25	0.00	0.38
Giugliano	21.0	5.66	1.27	14.0	0.01	0.02	0.07	0.72	0.00	0.40	0.40	0.12	0.53
Maddaloni	45.7	33.1	4.41	12.2	0.00	0.01	0.74	0.85	0.01	0.21	0.66	0.00	1.04
Teverola	38.8	91.0	7.58	32.9	0.10	0.13	0.33	1.81	0.19	3.99	0.57	0.01	1.17

Table 4. Mean concentrations (mg kg^{-1} ± SD, n=8) of elements in pre-exposed mosses (first column) and in the mosses exposed in the five municipalities (columns 2 - 6).

	Pre-exposure	**Giugliano**	**Teverola**	**Maddaloni**	**Acerra**	**Casal di Principe**
Al	*300 ± 75*	521 ± 65	638 ± 79	471 ± 59	508 ± 65	440 ± 41
As	*0.07 ± 0.05*	0.36 ± 0.07	0.24 ± 0.11	0.19 ± 0.09	0.11 ± 0.06	0.31 ± 0.14
Cd	*0.08 ± 0.017*	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.01
Cr	*2.25 ± 0.2*	2.99 ± 0.20	3.18 ± 0.19	2.94 ± 0.17	2.96 ± 0.13	2.83 ± 0.14
Cu	*4.77 ± 0.279*	7.54 ± 0.34	6.97 ± 0.35	6.72 ± 0.41	7.45 ± 0.23	5.86 ± 0.22
Fe	*403 ± 15*	574 ± 35	679 ± 53	557 ± 43	573 ± 30	535 ± 35
Hg	*48.2 ± 15*	45.8 ± 7.1	44.3 ± 7.2	46.0 ± 7.5	47.0 ± 7.2	58.9 ± 6.6
Ni	*0.83 ± 0.10*	1.13 ± 0.09	1.17 ± 0.12	1.08 ± 0.13	1.09 ± 0.07	1.14 ± 0.07
Pb	*0.57 ± 0.03*	2.38 ± 0.15	13.99 ± 1.64	3.14 ± 0.24	2.25 ± 0.08	2.22 ± 0.21
Se	*0.43 ± 0.07*	0.38 ± 0.08	0.26 ± 0.07	0.25 ± 0.08	0.36 ± 0.07	0.31 ± 0.08
Zn	*8.52 ± 0.7*	19.1 ± 1.1	17.2 ± 1.1	34.1 ± 16.4	24.9 ± 0.8	70.0 ± 9.4

Table 5. EC ratios of metals in mosses exposed in the five municipalities (the color indicates the accumulation/loss class according to Table 1).

	Giugliano	Teverola	Maddaloni	Acerra	Casal di Principe
Al	1.74	2.13	1.57	1.69	1.73
As	4.83	3.25	2.53	1.42	4.04
Cd	1.00	1.05	0.90	1.02	1.01
Cr	1.33	1.41	1.31	1.31	1.30
Cu	1.58	1.46	1.41	1.56	1.38
Fe	1.42	1.68	1.38	1.42	1.45
Hg	0.95	0.92	0.95	0.97	1.15
Ni	1.34	1.40	1.29	1.30	1.36
Pb	4.13	24.22	5.43	3.89	6.51
Se	0.88	0.59	0.57	0.83	0.74
Zn	2.24	2.02	4.00	2.92	7.91

Table 6. Matrix reporting the Pearson's r correlation values for moss uptake. Marked correlations are significant at $p < 0,05$; n=5.

	Al	As	Cr	Cu	Fe	Ni	Pb	Zn
Al	1	-0.05	0.99	0.46	0.99	0.6	0.9	-0.73
As		1	-0.09	-0.22	-0.08	0.58	-0.03	0.23
Cr			1	0.5	0.97	0.49	0.88	-0.79
Cu				1	0.33	-0.2	0.05	-0.91
Fe					1	0.62	0.96	-0.65
Ni						1	0.69	0
Pb							1	-0.42
Zn								1

Table 7. PAH content in pre exposed moss sample (T0) and mosses exposed in the five Municipalities.

	Total PAHs	PAHs 2-4 rings
T0	*0.064 ± 0.007*	*0.058 ± 0.007*
Giugliano	0.086 ± 0.006	0.070 ± 0.005
Teverola	0.076 ± 0.005	0.062 ± 0.005
Maddaloni	0.098 ± 0.019	0.077 ± 0.006
Acerra	0.098 ± 0.005	0.079 ± 0.005
Casal di Principe	0.090 ± 0.006	0.071 ± 0.005

Figures

Figure 1. Five Municipalities in Campania Region belonging to the RIPS area, chosen for emission inventory and biomonitoring data comparison.

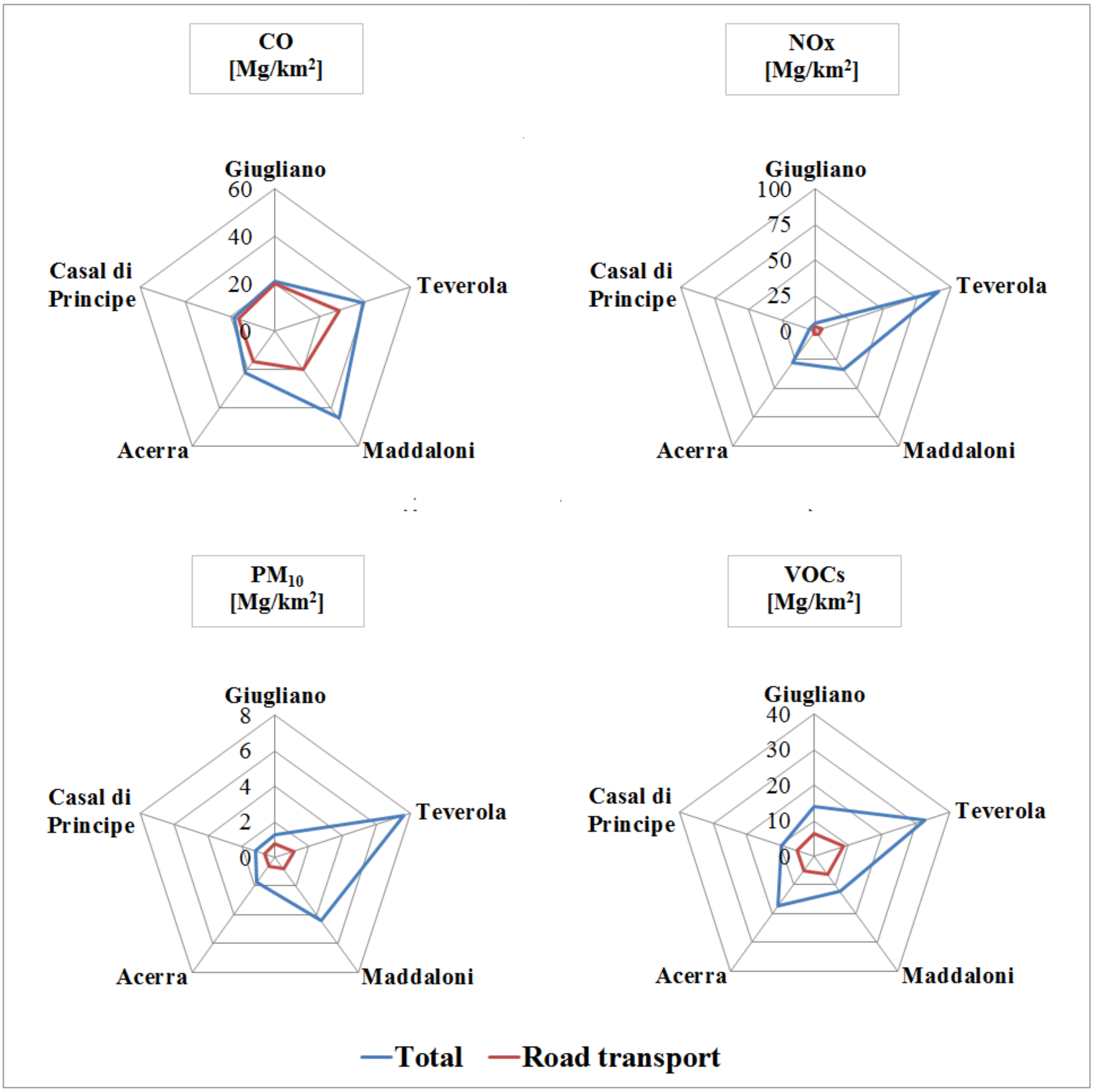


Figure 2. Total emissions and road transport contribution of each examined Municipality for the following pollutants: CO, NO$_X$, PM$_{10}$,VOCs.

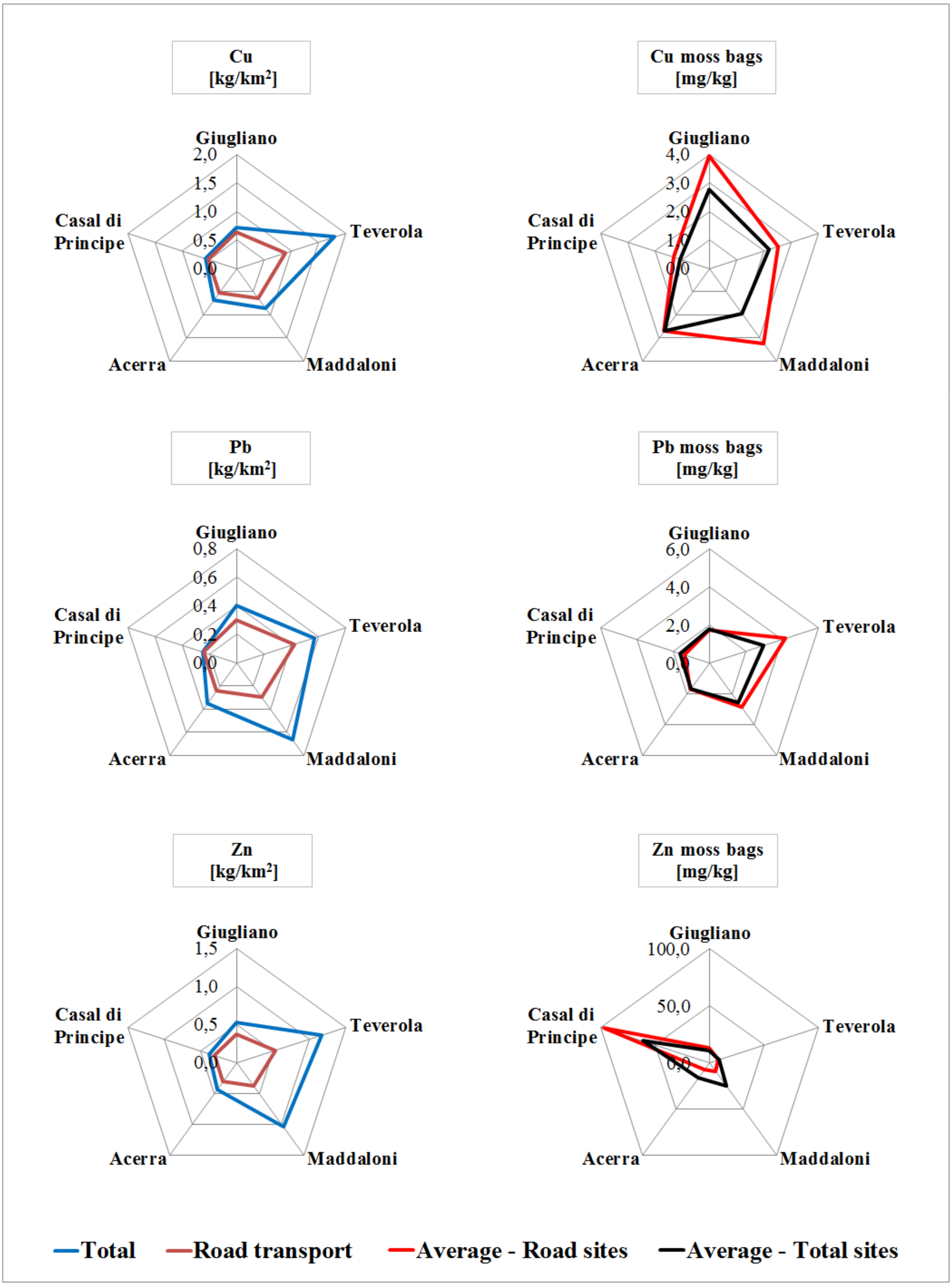


Figure 3. Total emissions and road transport contribution for Cu, Pb and Zn (left) and moss uptake of the same elements (right) at each Municipality.

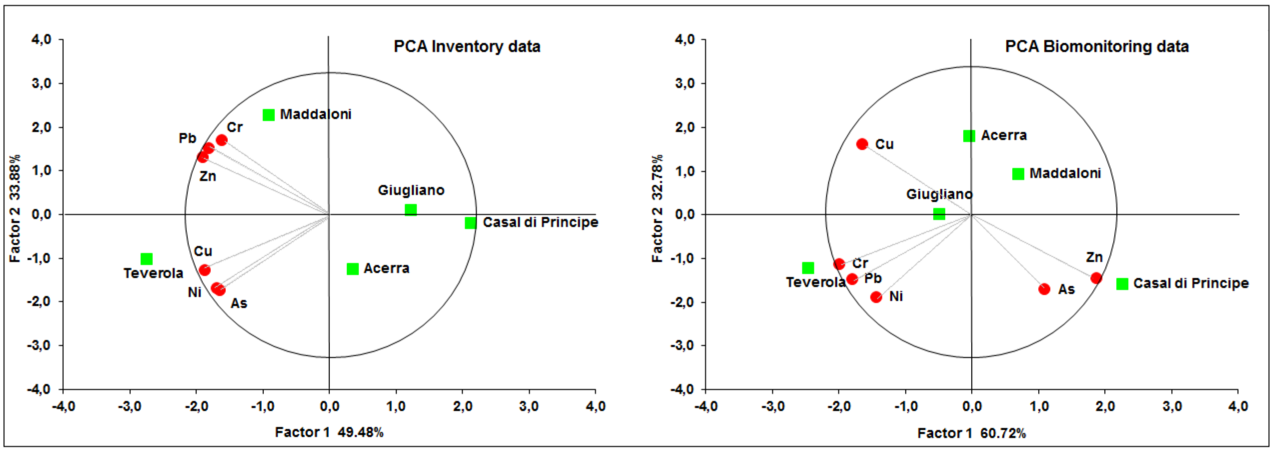


Figure 4. PCA of emission inventory metal data (left) and moss uptake (right) grouped by Municipality.

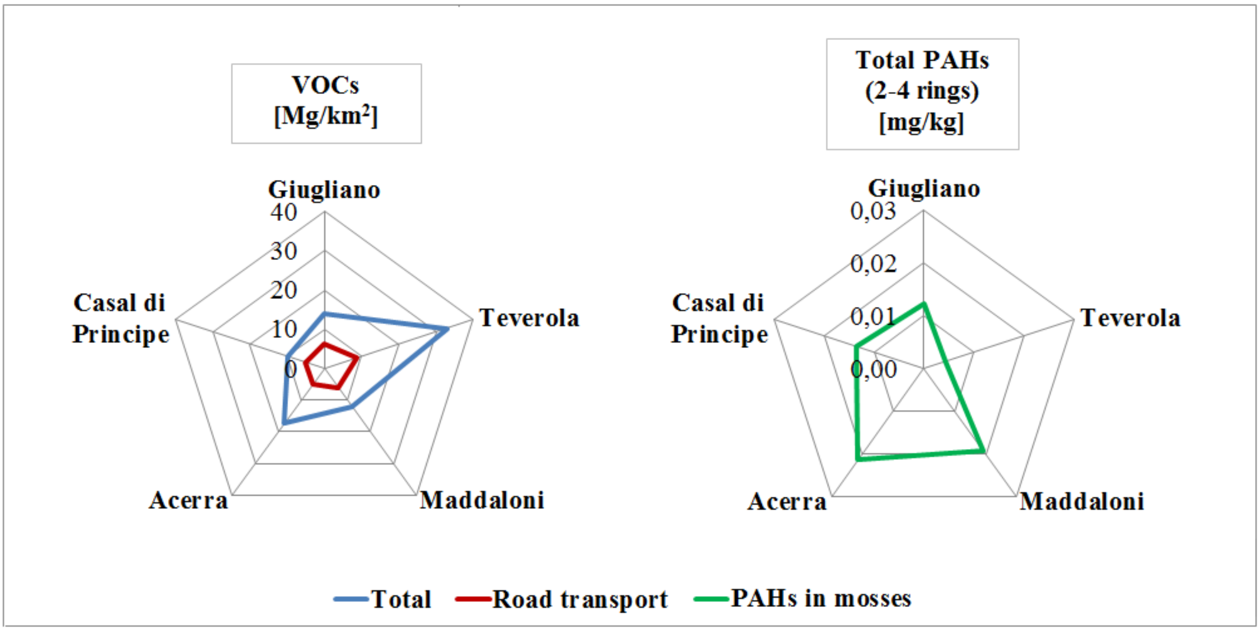
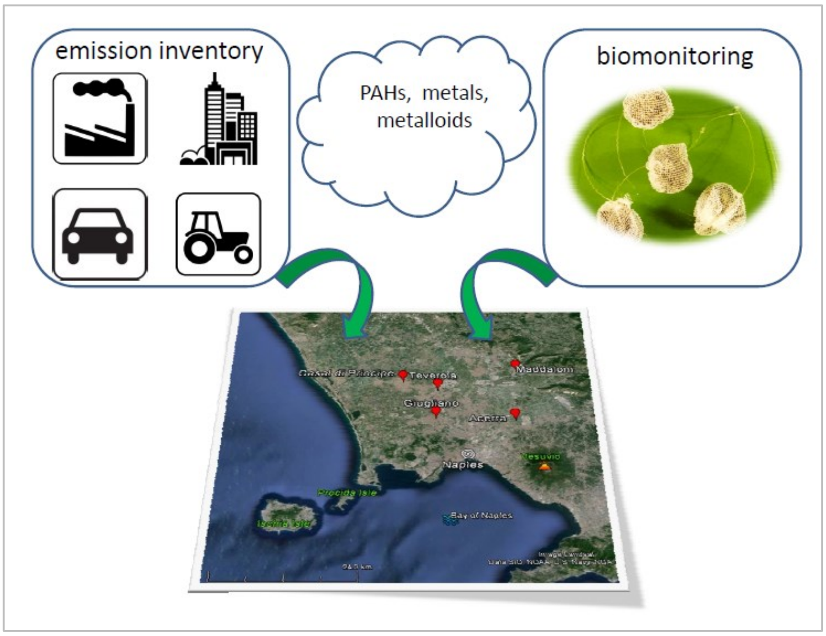


Figure 5. Total emissions and road transport contribution for VOCs (left) and moss uptake of PAHs (2-4 rings) (right) at each Municipality.

Graphical abstract



Chapter 4

Molecular and chemical characterization of a *Sphagnum palustre* clone: key steps towards a standardized and sustainable moss bag technique

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Abstract

This work aimed to define the molecular and chemical signature of a *S. palustre* clone developed in the framework of the EU-FP7 Mossclone project to improve the standardization and reliability of the moss-bag technique. The molecular characterization was performed by a set of DNA molecular markers (RAPD, ISJ, PCR-RFLP, sequencing and microsatellites) to tag the clone produced within the project. Molecular characterization also provided new DNA markers that can be applied in systematic analyses of *Sphagnum*, and gave new insights to implement well established techniques. The elemental composition of the clone was estimated by ICP-MS analysis of 54 major and trace elements, in relation to commonly applied pre-exposure treatments (moss oven devitalization and EDTA washing), and was compared with that of conspecific field moss. The devitalization pretreatment preserved the biomaterial and ensured its easy handling. Concentrations of almost all analyzed elements were significantly lower (from 10 to 100 times) in the clone than in field moss, apart from some elements (K, Mo, P and Na) deriving from the culture medium or EDTA treatment. A field test was performed for the first time, to compare the metal accumulation between bags filled with the clone and with *Pseudoscleropodium purum*. The *S. palustre* clone showed a significantly higher metal uptake performance, consistent with its physical-chemical and morphological properties. Therefore, the use of this biomaterial, with very homogenous morphological and chemical characteristics and a remarkable metal uptake capability, is strongly recommended in view of a rigorously standardized moss-bag protocol for the active monitoring of persistent atmospheric pollutants.

Keywords: *Atmospheric pollution, active biomonitoring, moss clone, trace elements, DNA molecular markers, Pseudoscleropodium purum*

Highlights

- A *Sphagnum palustre* clone is proposed as valid candidate for active biomonitoring
- A chemical and molecular characterization of the clone was performed
- Five anonymous sequences and their primers were obtained as new molecular markers
- Chemical content was stable and lower in the clone than in native *S. palustre*
- The uptake in the field was higher in the clone than in native moss *P. purum*

1. Introduction

Air pollution monitoring and management has been one of the main European scientific and political concerns since the 1970s. Three directives were adopted by EU for air quality assesment and management (1999/30/EC, 2002/3/EC, 2004/107/EC and 2008/50/EC) relating to metals, polycyclic aromatic hydrocarbons, ozone, sulphur dioxide, nitrogen oxides and dioxide, particulate matter in ambient air. Recently, a Clean Air Policy Package (CCEP-COM/2013/0918) was adopted on December 2013, with new air quality objectives up to 2030.

Mosses, either used as native species or as transplants (moss bags), can accumulate airborne inorganic and organic pollutants representing a cost effective and reliable tool, also combined with automatic monitoring devices and emission inventories (Adamo, 2008a; Spagnuolo et al., 2013; Harmens et al., 2015; Iodice et al., 2016). The active biomonitoring with moss bags allows to evaluate the atmospheric deposition of persistent air pollutants in a well constrained time period, in areas lacking native species such as urban and industrial environments. It has the great advantage that could be standardized at each step, from species selection to post-exposure treatments. As a rule, the moss exposed in bags is harvested in pristine areas; however, significant differences in baseline element contents and in accumulation performance exist among different species and even in the same species grown in different habitats, or in the same area but collected in different periods (e.g. Zechmeister et al., 2003; Couto et al., 2004; Tretiach et al., 2011). The standardization of the moss bag technique is one of the most pressing and crucial concern (Ares et al., 2012) and an essential prerequisite is the morphological and chemical homogeneity of the exposed material.

In the framework of the FP7 European project Mossclone, we firstly investigated the surface properties related to metal accumulation by four devitalized moss species widely used for biomonitoring purposes (González and Pokrovsky, 2014). *Sphagnum* sp. showed the highest uptake capability and afterwards, Beike et al. (2015) selected and axenically cloned *Sphagnum palustre* L., a species allowing in photobioreactors the production of a suitable biomass for bag preparation. Recently, the *Sphagnum* clone was studied in terms of adsorption capacity of Cu and Zn (González et al., 2016), revealing its promising use as biomaterial in moss-bag technique.

This work aimed to define the molecular and chemical signature of the *S. palustre* clone. The molecular characterization was performed by a set of DNA molecular markers to tag the clone. The elemental composition of the clone was estimated in relation to commonly applied pre-exposure treatments (oven devitalization and EDTA washing). A field test was also performed to compare the metal accumulation between bags filled with the clone and with *Pseudoscleropodium purum*, one of the most used species in passive and active biomonitoring surveys (e. g. Harmens et al., 2010; Ares et al., 2012). We tested for the first time the uptake ability of these two moss species after EDTA washing and oven devitalization. It is worth to note that only few works are focused on the comparison between different moss species used as transplants in bags (e.g. Culicov and Yurukova, 2006; Castello, 2007; Ares et al., 2014) and, in particular, none on the comparison between moss species devitalized after EDTA washing.

2. Materials and Methods

2.1 Molecular characterization

Two different lines of the cloned moss *S. palustre* named 2a and 12a (Beike et al., 2015), and a reference field sample (FS) collected in Posta Fibreno (central Italy, 41°41′42.69″N, 13°41′29.98″E, 290 m a.s.l.; Terracciano et al., 2012) were analyzed. In order to compose a clone-specific molecular tag we selected and applied several techniques among those suggested for molecular markers in mosses (e.g. Crespo Pardo et al., 2014). Although the highly preserved DNA of *Sphagnum* involves some difficulties in the detection of polymorphisms at sub-specific levels, three DNA regions were selected among barcoding candidate sequences suggested for mosses (Liu et al., 2010); in addition, both unilocus and multilocus techniques were applied.

Total genomic DNA was extracted using Dneasy Plant Mini Kit (Quiagen) following the manufacturer instructions. The different procedures for each technique are described below.

2.1.1. RAPD (Random Amplified Polymorphic DNA) and ISJs (Intron-exon splice junctions)

RAPD amplifications were performed according to the protocol reported in Skotniki et al. 1999, modified for the annealing temperature (40 °C instead of 35 °C). Two 5’-FAM (blue fluorophore) labeled primers (ISJ 04 and ISJ 10, see Sawicki and Szczecińska, 2007 for further details) were selected to obtain two characteristic multiband patterns. The reactions were performed in a final volume of 20 µl, containing 40 ng of genomic DNA, 1 U Taq polymerase, 10xPCR buffer (Fermentas, USA), 200 µM of each dNTP and 20 pmol of primer. The amplification protocol provided for a hot start (1 min at 94 ºC), followed by 44 cycles including the steps: denaturation at 94 ºC for 1 min, annealing at 52 ºC and 56 ºC for 1 min for the primers ISJ 04 and ISJ 10, respectively, and elongation at 72 ºC for 80 sec. A further final extension at 72 ºC for 5 min completed the PCR program. Amplification products were separated by capillary electrophoresis in an ABI Prism 3730 Genetic Analyzer (Applied Biosystem); fragments were visualized as an electropherogram profile and size determinations were made by GeneMapper ver. 3.1 Software (Applied Biosystem).

2.1.2 Sequences

The chloroplast regions *matK*, *rbcL* and *trnH-psbA* were amplified. The amplification products were purified (GFX PCR DNA and Gel Band Purification Kit - Amersham Biosciences - and sequenced by BigDye Terminator ver. 3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions. Sequence reactions were run in an ABI Prism 3730 Genetic Analyzer (Applied Biosystem); electropherograms were edited and aligned in Bioedit ver. 7.1 to obtain consensus sequences. The GenBank accession numbers of the sequences are respectively KJ865421, KJ865420 and KJ865419.

Five anonymous sequences were also developed by RAPD/ISJ reliable amplification products. Amplified bands were excised from the agarose gel and purified with the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences); fragments were ligated into a bacterial vector using TA Cloning Kit Dual Promoter – pCR II (Life Technologies) and used to transform *Escherichia coli* DH5α. After transformation, white colonies were picked and transferred to the PCR amplification mixtures (20 µl) and to a fresh LB plate for a replica.

2.1.3 Microsatellites

Fifteen primer pairs (Shaw et al., 2008), indicated as 1, 3, 4, 5, 9, 10, 14, 17, 18, 19, 20, 22, 28, 29 and 30, were used for microsatellite amplifications. According to the different size range of the products, one of the primer for each pair was 5'-FAM or 5'-HEX labeled and five different triple reactions were prepared and amplified following the experimental procedures described in Shaw et al., (2008). Amplification products were separated by capillary electrophoresis in an ABI Prism 3730 Genetic Analyzer (Applied Biosystem); fragment profile was visualized as an electropherogram by GeneMapper ver. 3.1 Software (Applied Biosystem).

2.1.4 PCR-RFLP

The anonymous DNA region RAPDf was amplified using the F-F and F-R primers and following the protocol reported in Shaw et al., 2003. The PCR products were purified by GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) and digested by a set of 17 restriction enzymes (Fermentas, Thermo Fisher Scientific) according to manufacturer's instructions. Amplified/digested products were visualized by an electrophoresis on 1.5 agarose gel.

2.2 Chemical characterization

2.2.1 Moss materials and pre-treatments

The elemental composition of *S. palustre* clone (line 12a) produced in photobioreactors (Beike et al., 2015) was determined in triplicate after oven drying at 40 °C for 8 hours (untreated clone, C-U) and after the following treatments: 1) EDTA washing and oven drying at 40 °C for 8 hours (C-EDTA);

2) oven devitalization by consecutive 8 hour-drying at 50, 80 and 100 °C (C-100); 3) EDTA washing and devitalization (C-EDTA100). The EDTA washing was performed as follows: 1 wash for 20 min with 10 mM EDTA (disodium salt di-hydrate, Panreac; 1l EDTA/12.5 g d.w. of moss) and 3 washes of 20 min each with distilled water (1l distilled water/10 g d.w. of moss).

Field samples of *S. palustre* from Posta Fibreno (see paragraph 2.1) were also analyzed. Moss shoots were mixed and washed with Milli-Q water (18 MΩ, Millipore, Bedford, MA, USA) to remove debris and soil particles. Only the green shoots (about 3-4 cm from the apical parts) were selected for the analysis, discarding brown or senescent tissues. Three subsamples of the water-washed field moss were dried at 40 °C for 8 hours (untreated field samples, FS-U) and other three were devitalized in oven as described above (FS-100).

2.2.2 Analytical determinations

Sphagnum palustre field and clone samples were acid digested in a microwave (MARS 5 system CEM) in ISO 2 workstations in the Géosciences Environnement Toulouse (G.E.T., Toulouse, France) laboratory cleaning room (class A 10,000). Moss sample (0.1 g d.w. each) were mixed with 9 ml bi-distilled HNO_3, 0.2 ml suprapure HF (Merck KGaA, Darmstadt, Germany) and 1 ml suprapure H_2O_2 (Merck KGaA, Darmstadt, Germany) in 20 ml Teflon containers (Savilex®). A one-stage digestion procedure consisting of a 20 minute-holding stage at 150 °C, 1600 W and 100 psi was applied. After cooling, the mineralized solutions were evaporated at 70 °C for 24 hours on a hot plate and the residue dissolved by sonication in 20 ml of 2 % HNO_3. The elemental analysis was carried out by ICP-MS using an Agilent 7500 ce (Agilent Technologies, Santa Clara, California, USA). The concentrations of 54 elements, including rare earths, were evaluated as the mean of 100-times scanned measurements. Details about the entire analytical procedure are available in Viers et al. (2007, 2013) and Stepanova et al. (2015).

2.2.3 Procedure control

Mineralization solutions without moss samples (i.e. blanks) were used as negative control (1 blank every 8 samples) to ensure no contamination from the acid digestion. The concentrations of 16 elements (Cd, Co, Dy, Eu, Ga, Gd, Hf, Ho, Lu, Mg, Na, Nb, Ni, P, Sn, Ta, Th, Tl, Tm and W) in the blanks were always below the detection limits. Erbium, Tb, U, Yb ranged between 0,1-1,0 ng/l. Beryllium, Ce, Cs, Ge, La, Nd, Pr, Sb, Sm, Y, Zr between 1,0-10 ng/l. Aluminium, As, B, Ba, Ca, Cr, Cu, Fe, Li, Mn, Mo, Pb, Pr, Rb, Sr, Te, Ti, V, Zn >10 ng/l. Element concentration data measured in moss samples were always calculated by subtracting blank values.

Reference standard material BCR-482 was employed (1 control each digestion batch) to check the accuracy and precision of the analytical procedure. Data quality control was assessed by comparing the certified and measured values for BCR 482 reference material in terms of recovery (%), and by checking the precision of ICP analysis by the relative percentage differences (RPD) and the relative standard deviation (RSD) among the reference material replicates. For all the elements measured in the reference material, the recovery ranged between 71 % (Cr) and 92 % (Al) with only one lower value for Cu (64 %). The RPD was about 20 % for most elements, with the exception of Sn (61 %), Ta (53 %) and W (49 %). The precision of ICP analysis was considered acceptable, with the RSD values lower than 10 %, apart for Te (about 30 %).

2.3 Field test

Bags with the *S. palustre* clone (C-EDTA100) and *P. purum* (Hedw.) M. Fleisch were exposed in the field to compare their metal uptake ability (due to European restrictions for species protection 92/43/EEC, it was avoided the massive collection of naturally growing *S. palustre*). Green apical parts (5 cm) of *P. purum* gametophytes, collected in a pristine area of Galicia (NW Spain), were cut from shoots, washed with Milli-Q water to remove exogenous particles and devitalized through EDTA treatments (section 2.2.1.).

Three grams of moss were exposed in nylon bags (spherical shape, 2 mm net mesh size, ø ~11 cm), in triplicate for six weeks at 4 m above the ground, using nylon strings linked to polyethylene rods. Ten exposure sites with different atmospheric contamination levels were selected in urban, industrial agricultural and background areas of Italy and Spain. After the exposure, mosses were removed from nylon bags, oven-dried at 40 ºC until constant weight and homogenized into a fine powder (< 150 µm) at 600 rpm and 15 min by a planetary mill (RETSCH PM 200) equipped with agate bowls. Aluminum, Ba, Cr, Cu, Fe, Pb, Sr and Zn concentrations were determined in acid-digested samples by ICP-MS, following the protocol described in the section 2.2.1. Ten unexposed samples of the clone and *P. purum* were also analyzed to assess metal concentrations before the exposure.

2.4 Data processing

The element concentrations of all moss samples were evaluated as the average of three replicates for each sample and reported on a dry weight basis. All data were processed using Microsoft Excel, STATISTICA ver. 7 and the free R software ver. 3.2.2.

The non-parametric Kruskal Wallis test was performed to check significant differences in chemical composition between materials and treatments. The Nemenyi test was used as *post hoc*, according to Zar (2010), who suggests this test for comparison of groups with an equal number of data.

Multivariate exploratory analysis was applied to the element concentrations in moss samples. All the data were clustered after standardization of the variables. The principal component analysis (PCA) was also performed (Podani, 2000).

The Wilcoxon matched pairs test (Lehmann, 1975) was used to evaluate the significance of differences between element uptake in *S. palustre* clone and in *P. purum*.

3. Results and Discussion

3.1 Moss clone molecular characterization

Comparisons among different DNA extractions of the *Sphagnum* clone 12a showed that some RAPD primers (OPB 15 and OPJ 19) provided reproducible multiband patterns (Figure 1).

The RAPD technique is generally considered poorly reliable because the shortness of the primers allows for the annealing to any DNA template eventually contaminating the sample. However, this problem is strongly reduced in axenic plant material; moreover, the used annealing temperature of 40 °C (i.e. 5 °C higher than that in the original amplification protocol proposed by Skotniki et al., 1999), greatly enhanced the stringency of the reaction and produced very constant banding patterns.

The amplification of *S. palustre* DNA from cloned and field moss samples by ISJ primers provided the fragments reported in Table 1.

These primers, already tested in mosses, including those of the genus *Sphagnum* (Sawicki et al. 2009, Sawicki and Szczecińska, 2011), clearly distinguished different taxa and at within-species level. The primers are designed partly complementary to DNA region at the junction between intron and exon; as a consequence, choosing specific primer pairs, introns or exons should be amplified, at least in theory. But such characteristics do not avoid primer annealing in different regions, according to base pair complementarities. To counterbalance this drawback fragment separation was carried out by capillary electrophoresis in order to enhance the reliability of the procedure and to assign a precise length to each fragment.

In addition to *matK*, *rbcL* and *trnH-psbA* regions (GeneBank accession codes KJ865419, KJ865420, KJ865421 for *S. palustre* clone 12a), five anonymous regions were developed and appropriate primer pairs were designed and tested in the clone (GeneBank accession codes KP889208, KP889209, KP889210, KP965888, KP965889). Primer sequences, with their annealing temperature and the expected size of each region, are given in Table 2. BLAST analysis against the GenBank database did not provide positive hits, confirming the anonymous nature of the five regions. Considering the highly conserved genome of *Sphagnum*, these novel sequences should provide high resolution as SCAR (Sequence Characterized Amplified Region) markers, for detecting polymorphisms in systematic studies.

As for PCR-RFLP, a technique already applied in mosses (Vanderpoorten et al., 2003), several nuclear and plastid DNA regions (ITS, PsbC-TrnS and TrnF-V1) were tested by a set of 17 restriction enzymes before the anonymous regions RAPDa, RAPDb and RAPDf (Shaw et al., 2003), but no polymorphism was found. The double digestion of RAPDf with *Hinf*I - *Hind*III derived in a characteristic, reproducible band pattern for the clone (Figure 2, see arrows). No polymorphism and/or not reproducible multiband patterns were provided by the other restriction endonucleases.

Microsatellite analysis produced four polymorphisms between the clone and field shoots (Table 3) at the loci 5, 9, 14 and 17; a polymorphism was also observed between the two clone lines analyzed, at the locus 28.

3.2 Elemental signature of the clone and field samples

The elemental composition of field *S. palustre* (FS) and the clone (C) are reported in Table 4.

In general, regardless of treatment, most element concentrations were significantly lower ($p < 0.05$) in the clone compared to the field moss. In the latter the concentrations of Al, Ba, Cs, Hf, Nb, Pb, Ta, Y and rare earth elements were two order of magnitude higher and more than 10 times higher for almost all the other elements. The clone had significantly higher ($p< 0.05$) K, Mo, P concentrations, likely due to their occurrence in the culture medium, and higher Na concentrations in EDTA treated samples (C-EDTA and C-EDTA100; Table 4). The high concentrations of some typical soil elements (i.e., Al, Fe, Ti, Ca and Mg) in the field samples might indicate the contribution of soil dust to the moss chemical composition (Bargagli, 1998; Adamo et al., 2008b).

Devitalization is a very useful option for active biomonitoring with mosses because bryophytes maintain a remarkable metal uptake capability and the lack of metabolic activity during the exposure reduces the variability of the results (Adamo et al., 2007; Giordano et al., 2009; Fernández et al., 2010). The oven devitalization, as well as the EDTA treatment, did not produce any substantial changes in the elemental composition of all moss samples; no statistically significant differences were observed between FS-U and FS-100, and between C-U and C-100 or in the *S. palustre* clone with and without EDTA treatment. The treatment with EDTA, a well-known chelating agent, useful to decrease metal concentrations in field mosses and to increase their cation exchange capability before the exposure (e.g. Lodenius and Tulisalo, 1984; Ferreira et al., 2009; Chen et al., 2015; Iodice et al., 2016), had no evident effect on the clone elemental composition. This was likely due to very low element concentrations in the biomaterial. As the EDTA washing added Na and induced morphological damages to the moss clone, making shoots and leaves very fragile (see SEM micrographs in Figure 3) this treatment can be avoided making the set-up of clone bags more convenient and trouble-free.

The Cluster Analysis of the element concentration data related to FS and C samples (Figure 4) revealed two main clusters (a and b) which clearly separated field *Sphagnum* samples from the clone ones. Both clusters were divided in sub-clusters generally according to the different treatments, even if the between-group variance was not significant.

The ordination of moss samples by PCA (Figure 5) distinguished clone from field samples along the factor 1, which explains a good 86 % of the total variance. Along the factor 2, EDTA-treated samples were separated from the other clones, however the low total variance (9 %) accounted for a chemical homogeneity of all clone samples. Most of the element vectors are oriented towards the field-moss group, leaving in the clone area only those elements derived from the culture medium (i.e. B, K, Mo, P and Zn) or from the EDTA treatment (Na). Probably, an additional rinsing by distilled water might help to wash out the elements deriving from culture medium.

Although comparisons with literature data on the elemental composition of other *Sphagnum* species are made difficult by differences in sample preparation and chemical digestion procedures, the treated and untreated samples of *S. palustre* clone showed the lowest element concentrations among other *Sphagnum* species collected from reference areas of the world (Table S1). The only exceptions were *S. cristatum* from New Zealand (washed with conc. HNO_3 before the exposure; Archibold and Crisp, 1983) and *S. girgensonii* from Russia (Aničić et al., 2009b). The acid washing was used as devitalizing treatment for mosses especially in the past; recent studies (e.g. Adamo et al., 2007; Tretiach et al., 2007) suggest the use of other methods making treated moss shoots less fragile and reducing the loss of material during the exposure.

The moss clone grown in bioreactors with constant temperature, pH, light and composition of the culture medium (Beike et al., 2015) had a much more homogenous chemical composition than that of field moss, which is affected by changes in climatic conditions, element bioavailability, metabolic activity and growth rate (Bates, 2000; Stepanova et al., 2015).

3.3 Field test

The Figure 6 shows the Al, Ba, Cr, Cu, Fe, Pb, Sr and Zn accumulation (defined as the difference in element concentration before and after exposure) by *S. palustre* clone and *P. purum* in ten Italian and Spanish exposure sites.

The values were expressed by averaging, for each element, all the concentrations measured in all the exposure sites. We only considered the elements showing a post-exposure concentration higher than $C_{T0} + 2*SD_{T0}$, (where C and SD are the concentration and the standard deviation for a given element in pre-exposure (T0) moss; Couto et al., 2004) in at least 60 % of the samples. The *S. palustre* clone, exposed in bags for the first time, showed a significantly higher ($p< 0.05$) metal uptake performance compared to the naturally growing *P. purum*. In the two species the accumulation of Al, Fe and Zn differed for about one order of magnitude, and 2-3 times for Ba, Cr, Cu, Pb, Sr. Differences in metal accumulation capacity were also found by Ares et al. (2014) who, comparing naturally growing *Sphagnum denticulatum* L. and *P. purum* (transplanted in bags without any EDTA pretreatment), found a higher uptake ability in *S. denticulatum*. These remarkable diversities between the two tested species were likely due to differences in specific surface area (SSA), cationic exchange capacity, binding sites and metal uptake capacity, especially considering that the devitalization eliminates any metabolic contribution to the uptake. The *S. palustre* clone has a SSA = 28 ± 1 m^2 g^{-1} d.w., with 0.65 mmol g^{-1} proton binding sites mainly as carboxylic and phenolic groups (González et al., 2016); in addition, González and Pokrovsky (2014) found that *Sphagnum* sp. has the highest proton and metal adsorption capacity in comparison to *P. purum*, *Brachytecium rutabulum* and *Hypnum* sp. Moreover, *Sphagnum* genus displays morphological features (i.e. hyalocysts, empty and dead cells with a variable number of pores) making it particularly suitable for the outside or inside cell wall uptake of airborne particulate matter (Giordano et al., 2005; Vingiani et al., 2004, 2015).

4. Conclusions

Molecular analyses based on unilocus and multilocus DNA markers characterized *S. palustre* clone developed within the Mossclone Consortium, with the intent to tag the clone. This step also provided new DNA markers that can be applied in systematic analyses of *Sphagnum*, and gave new insights to implement well known techniques for molecular analyses of mosses. Comparisons among the elemental concentration of *S. palustre* naturally growing in background areas and that of differently treated clones showed that the latter have much lower and homogenous element concentrations, providing an excellent biomaterial for the active monitoring of persistent air pollutants. The concentrations of 54 elements were determined and only those of K, Mo and P were higher in the clone; these elements derive from the culture medium and their content can probably be reduced through pre-exposure additional water rinsing. Due to very low element concentrations, near or under detection limits for rare earths, this biomaterial seems particularly suitable to monitor atmospheric depositions also in low polluted environments and for short exposure periods. Although these properties are independent of the clone pre-treatments, we recommend the devitalization as a key pre-treatment step because it ensures a standardized biomaterial "ready to use". The trial in the field revealed the capacity of *S. palustre* clone to uptake airborne elements, even better than the widely used naturally growing *P. purum*. Therefore, we encourage the use of this biomaterial, with low and stable elemental signature, in air pollution biomonitoring, in the view of a completely standardized moss-bag protocol.

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Tables

Table 1. ISJ analysis of the *S. palustre* clone (lines 2a and 12a) compared to the field sample. Fragment length is given in bp.

Primer	**2a**	**12a**	**FS**
ISJ 4	44	44	-
	76	76	-
	132	132	132
ISJ 10	70	70	-
	110	110	-
	132	132	132
	166	166	-
	175	175	-
	187	187	-

Table 2. Primer sequences of the SCAR markers; for each primer pair the annealing temperature (Tm) and the expected size (Es) of the amplification product are indicated.

Primer	**Sequence (5’-3’)**	**Tm**	**Es (bp)**
S40_fw	TTTTCCACATACACCACCGC	58.8	350
S40_rv	AGTTAACGTTACCCAGGCGA	59.0	
S42_fw	ACGTCGGCTCTCAGGTATTC	59.3	400
S42_rv	CTTCGTTGTGGGGTCTGTTG	59.1	
S44_fw	GCAGTAATTGATCTTGGCAACC	58.2	250
S44_rv	TGCACTGCCAAAAGTTTCAG	57.4	
T31_fw	ACCACCACCACGCATAGAG	59.4	430
T31_rv	AAATGTGTTGAAGACCCCATGA	58.2	
T37_fw	CGCATTCACAGGGCTCTAAC	59.0	580
T37_rv	AGCTTGTAACGAAGGGACCT	58.7	

Table 3. Microsatellite analysis of the *S. palustre* clone (lines 2a and 12a) compared to the field sample. Fragment length is given in bp and length polymorphism are in bold.

Locus (repeat motif)	***Sample***		
	2a	***12a***	***FS***
1 (CA)	244-254	244-254	244-254
3 (CA)	169	169	169
5 (GT)	**192-198**	**192-198**	**188-192**
9 (CT)	**159-174**	**159-174**	**169-184**
10 (GA)	233	233	233
14 (AG)	**228**	**228**	**214**
17 (AAG)	**159**	**159**	**162**
19 (AAG)	246-267	246-267	246-267
20 (TTC)	264-289	264-289	264-289
22 (GAT)	99-102	99-102	99-102
28 (AC)	**225-237**	**225-235**	**225-235**
29 (AAG)	194-197	194-197	194-197
30 (GAT)	139-142	139-142	139-142

Table 4. Mean elemental concentrations (mg kg^{-1} ± SD, n = 3) of *S. palustre* samples. FS = field moss; C = clone; U = untreated samples; 100 = devitalized samples; EDTA = EDTA-treated moss; n. d. = not determined; ≤ d. l. = concentrations under detection limit.

	FS-U	**FS-100**	**C-U**	**C-100**	**C-EDTA**	**C-EDTA100**
Al	1017 ± 10	953 ± 57	4.9 ± 0.3	55.9 ± 0.7	6.4 ± 1.6	1.8 ± 0.3
As	0.29 ± 0.02	0.25 ± 0.03	0.021 ± 0.003	0.018 ± 0.002	0.016 ± 0.002	0.015 ± 0.002
B	2.8 ± 0.5	12.4 ± 0.3	6.9 ± 0.7	13.3 ± 0.3	1.8 ± 0.2	2.0 ± 0.2
Ba	17.8 ± 1.0	17.8 ± 1.4	0.06 ± 0.01	0.199 ± 0.006	0.30 ± 0.08	0.28 ± 0.04
Be	0.04 ± 0.003	0.035 ± 0.005	0.00169 ± 0.00001	0.0022 ± 0.0001	n. d.	n. d.
Cd	0.11 ± 0.01	0.07 ± 0.01	0.007 ± 0.001	0.006 ± 0.001	0.004 ± 0.001	0.003 ± 0.0002
Co	0.32 ± 0.03	0.30 ± 0.04	0.31 ± 0.02	0.30 ± 0.02	0.010 ± 0.001	0.0096 ± 0.0004
Cr	1.3 ± 0.1	1.3 ± 0.3	0.04 ± 0.01	0.22 ± 0.01	0.0383 ± 0.0004	0.07 ± 0.07
Cs	0.23 ± 0.02	0.20 ± 0.04	0.00039 ± 0.00004	0.0026 ± 0.0001	0.003 ± 0.001	0.0016 ± 0.0001
Cu	3.1 ± 0.2	3.0 ± 0.5	0.79 ± 0.08	0.79 ± 0.03	0.83 ± 0.08	0.82 ± 0.05
Fe	442 ± 1	391 ± 27	109 ± 18	111 ± 7	79.8 ± 2.6	93.4 ± 0.7
Ga	0.21 ± 0.01	0.19 ± 0.03	0.010 ± 0.001	0.01 ± 0.00	0.007 ± 0.004	0.0032 ± 0.0001
Ge	0.024 ± 0.002	0.035 ± 0.005	0.0007 ± 0.0002	0.00056 ± 0.00004	0.0007 ± 0.0003	0.0009 ± 0.0002
Hf	0.046 ± 0.003	0.04 ± 0.01	0.0010 ± 0.0002	0.0008 ± 0.0001	0.0005 ± 0.0002	0.00029 ± 0.00003
Li	0.51 ± 0.04	0.43 ± 0.08	0.010 ± 0.001	0.032 ± 0.001	0.03 ± 0.02	0.0232 ± 0.0002
Mn	60.3 ± 3.6	48.4 ± 5.9	109 ± 6	103 ± 5	6.5 ± 0.4	6.2 ± 0.2
Mo	0.11 ± 0.01	0.09 ± 0.02	3.1 ± 0.6	2.95 ± 0.12	3.0 ± 0.4	2.8 ± 0.2
Na	693 ± 1	656 ± 37	13.6 ± 1.0	20.2 ± 0.6	1867 ± 11	1845 ± 46
Nb	0.23 ± 0.03	0.19 ± 0.05	0.0008 ± 0.0001	0.0006 ± 0.0001	0.0005 ± 0.0003	0.0003 ± 0.0001
Ni	1.1 ± 0.1	1.0 ± 0.2	0.11 ± 0.03	0.16 ± 0.06	0.06 ± 0.03	0.55 ± 0.33
Pb	2.0 ± 0.2	1.9 ± 0.4	0.016 ± 0.002	0.05 ± 0.02	0.02 ± 0.01	0.006 ± 0.001
Rb	6.9 ± 0.5	5.2 ± 0.9	0.39 ± 0.03	0.38 ± 0.01	0.37 ± 0.02	0.36 ± 0.02
Sb	0.16 ± 0.02	0.18 ± 0.02	0.006 ± 0.002	0.017 ± 0.003	0.004 ± 0.001	0.06 ± 0.03
Sn	1.1 ± 0.2	0.58 ± 0.12	0.020 ± 0.003	0.013 ± 0.001	0.16 ± 0.01	0.17 ± 0.02
Sr	24.5 ± 1.1	21.9 ± 3.5	1.7 ± 0.1	2.6 ± 0.1	1.55 ± 0.05	1.6 ± 0.1
Ta	0.014 ± 0.002	0.011 ± 0.003	0.00013 ± 0.00002	0.00009 ± 0.00003	0.00009 ± 0.00004	0.00008 ± 0.00002
Te	≤ d. l.	≤ d. l.	n. d.	≤ d. l.	n. d.	n. d.
Ti	22.7 ± 1.8	18.6 ± 3.6	0.89 ± 0.11	0.81 ± 0.01	0.76 ± 0.06	0.68 ± 0.05
Tl	0.025 ± 0.003	0.019 ± 0.004	0.013 ± 0.001	0.013 ± 0.004	0.010 ± 0.001	0.0113 ± 0.0003
V	1.8 ± 0.1	1.5 ± 0.3	0.046 ± 0.004	0.061 ± 0.002	0.05 ± 0.02	0.040 ± 0.002
W	0.030 ± 0.003	0.02 ± 0.01	0.006 ± 0.002	0.0047 ± 0.0005	0.004 ± 0.001	0.0037 ± 0.0004
Zn	24.2 ± 1.2	20.6 ± 3.5	51.7 ± 3.1	51.0 ± 1.5	8.5 ± 0.3	9.2 ± 0.4
Zr	1.6 ± 0.1	1.3 ± 0.2	0.05 ± 0.01	0.043 ± 0.003	0.03 ± 0.01	0.019 ± 0.003

Table 4. Continued.

	FS-U	**FS-100**	**C-U**	**C100**	**C-EDTA**	**C-EDTA100**
macronutrients						
Ca	9121 ± 84	7140 ± 403	6950 ± 130	6719 ± 86	2446 ± 46	2245 ± 32
K	2692 ± 42	2262 ± 160	12647 ± 159	12228 ± 267	9465 ± 78	9135 ± 289
Mg	2069 ± 26	1443 ± 68	1095 ± 0.5	1.018 ± 40	887.5 ± 1.8	879 ± 12
P	258.4 ± 10.5	292.3 ± 9.4	2175 ± 63	1931 ± 26	1716 ± 9	1543 ± 11
Actinoids and Rare Earths						
Ce	1.2 ± 0.1	1.0 ± 0.2	0.002 ± 0.001	0.0075 ± 0.0003	0.0012 ± 0.0001	0.0026 ± 0.0004
Dy	0.07 ± 0.01	0.06 ± 0.01	0.00015 ± 0.00002	0.00055 ± 0.00007	0.00015 ± 0.00002	n. d.
Er	0.034 ± 0.004	0.030 ± 0.005	0.00010 ± 0.00003	0.0002 ± 0.0001	0.00009 ± 0.00001	0.0001 ± 0.0001
Eu	0.022 ± 0.002	0.018 ± 0.003	0.00005 ± 0.00003	0.00019 ± 0.00002	0.000059 ± 0.000004	0.0001 ± 0.0001
Gd	0.09 ± 0.01	0.08 ± 0.02	n. d.	0.0008 ± 0.0002	0.00022 ± 0.00002	n. d.
Ho	0.012 ± 0.001	0.011 ± 0.002	≤ d. l.	0.00008 ± 0.00003	≤ d. l.	≤ d. l.
La	0.64 ± 0.06	0.55 ± 0.10	0.002 ± 0.001	0.005 ± 0.001	0.0010 ± 0.0002	0.001 ± 0.001
Lu	0.0044 ± 0.0004	0.004 ± 0.001	≤ d. l.	0.00006 ± 0.00002	≤ d. l.	n. d.
Nd	0.52 ± 0.05	0.67 ± 0.15	0.0016 ± 0.0002	0.0033 ± 0.0002	0.0006 ± 0.0001	0.001 ± 0.001
Pr	0.15 ± 0.01	0.12 ± 0.02	0.0004 ± 0.0001	0.0009 ± 0.0001	0.00018 ± 0.00004	0.0002 ± 0.0001
Sm	0.10 ± 0.01	0.09 ± 0.01	0.0004 ± 0.0001	0.0008 ± 0.0001	0.001 ± 0.001	0.0002 ± 0.0002
Tb	0.012 ± 0.001	0.011 ± 0.002	0.00004 ± 0.00001	0.00010 ± 0.00002	≤ d. l.	≤ d. l.
Th	0.12 ± 0.03	0.122 ± 0.029	0.009 ± 0.004	0.013 ± 0.005	0.006 ± 0.002	0.009 ± 0.006
Tm	0.0047 ± 0.0003	0.004 ± 0.001	n. d.	0.00005 ± 0.00002	0.00005 ± 0.00001	n. d.
U	0.05 ± 0.01	0.04 ± 0.01	0.003 ± 0.001	0.0030 ± 0.0001	0.003 ± 0.001	0.0032 ± 0.0004
Y	0.33 ± 0.02	0.29 ± 0.05	0.0009 ± 0.0002	0.0025 ± 0.0001	0.0006 ± 0.0001	0.0006 ± 0.0002
Yb	0.030 ± 0.002	0.027 ± 0.005	0.0002 ± 0.0001	0.0003 ± 0.0001	n. d.	0.00016 ± 0.00002

Figures

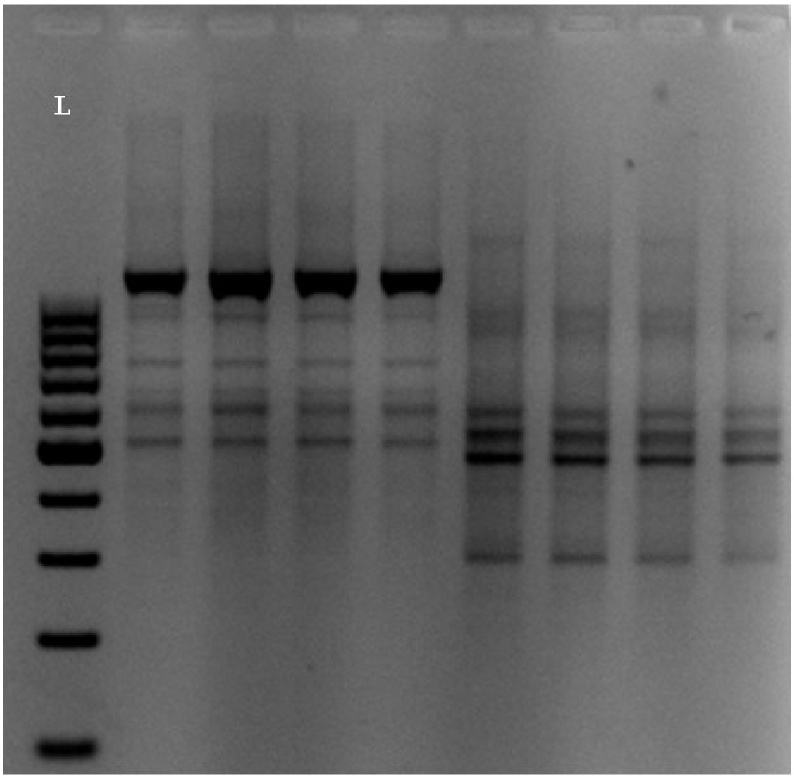

Figure 1. RAPD amplification of the clone 12a; L = ladder 100 bp; lanes 2 to 5: DNA amplifications of 4 different shoots by OPB15 primer; lanes 6 to 9: DNA amplifications of 4 different shoots by OPJ19 primer.

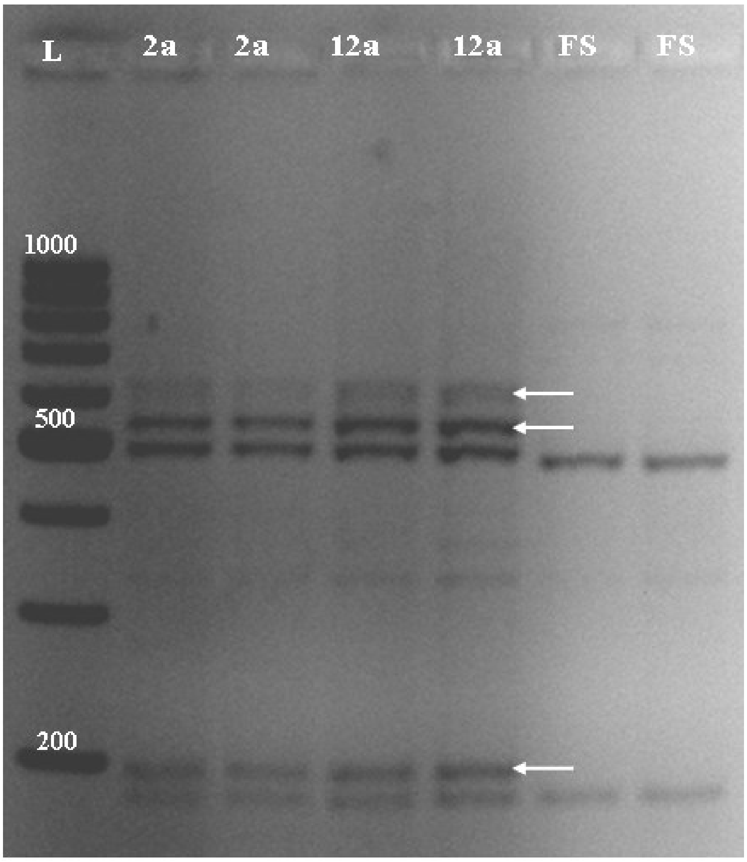

Figure 2. Double digestion by Hind III/HinfI of RAPDf DNA region. L = ladder 100 bp; 2a and 12a are two different lines of *S. palustre* clone; FS = field shoots. Each digestion was performed on a different DNA extractions.

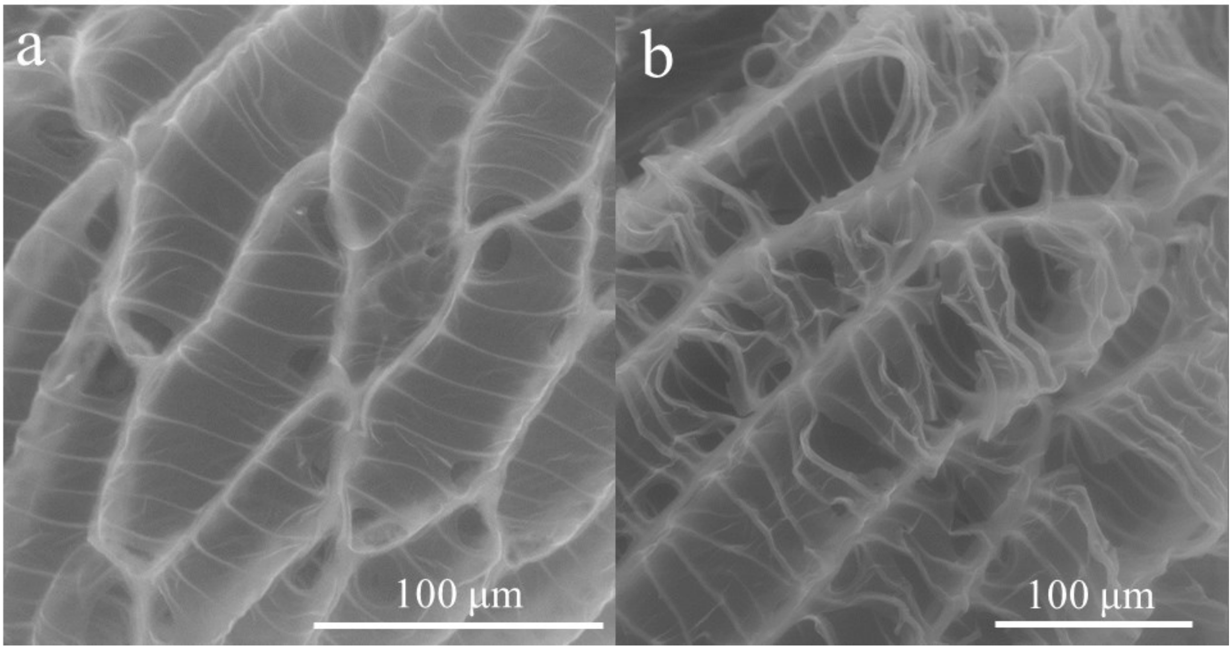

Figure 3. Scanning electron micrographs of *S. palustre* clone before (a) and after EDTA treatment (b).

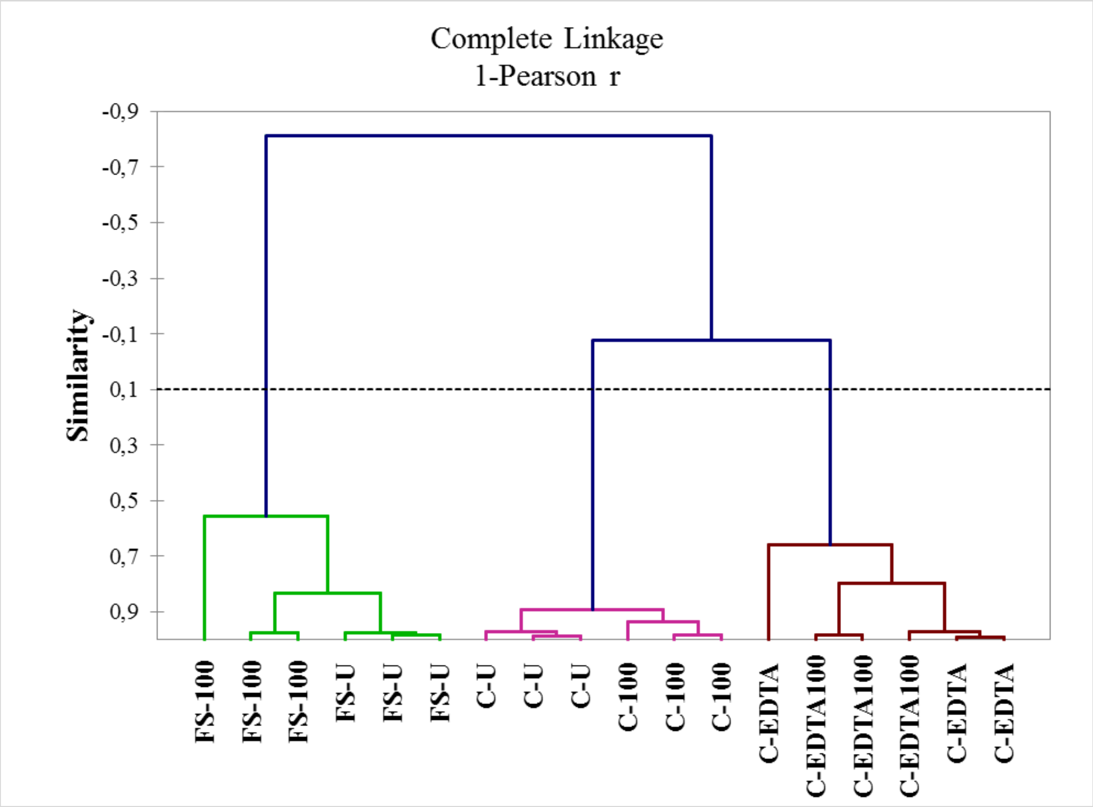

Figure 4. Cluster Analysis of the element concentration data related to FS and C *Sphagnum* samples. For the labels see paragraph 2.2.1.

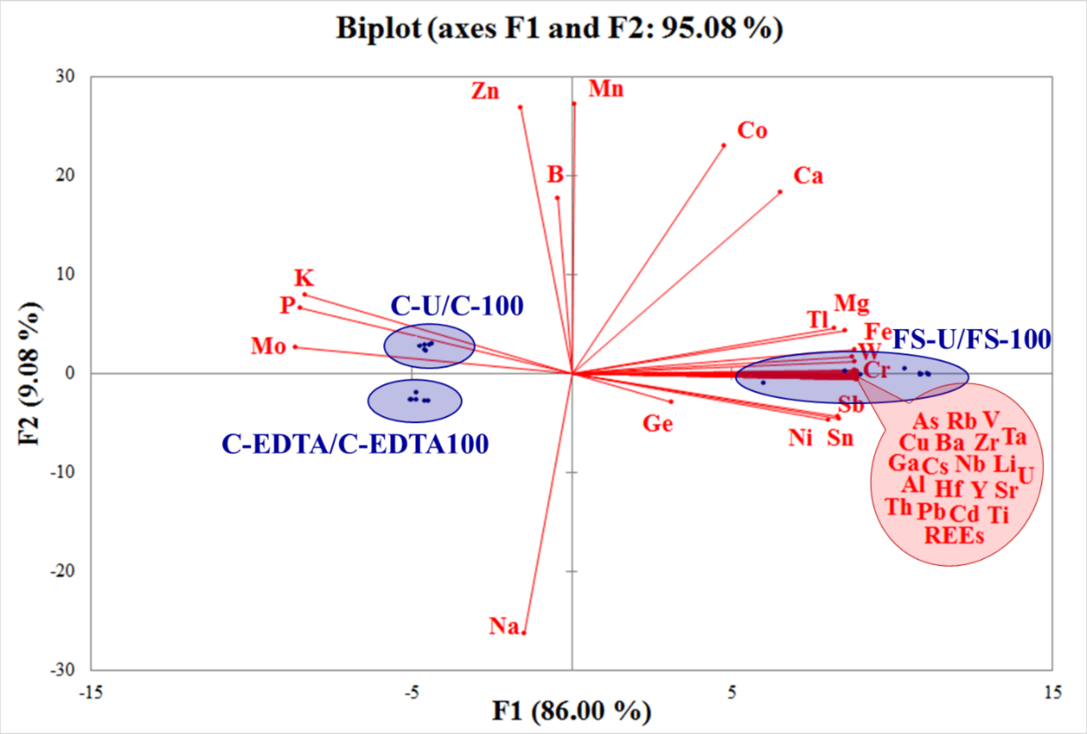

Figure 5. Biplot of Principal Component Analysis for FS and C *S. palustre* data. For the label-codes see paragraph 2.2.1.

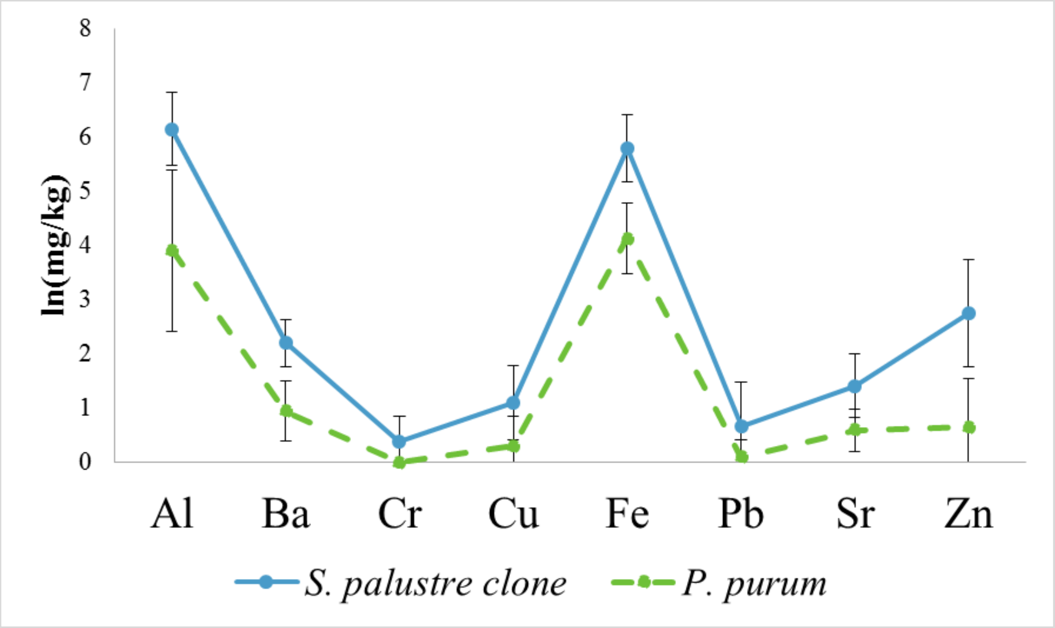

Figure 6. Accumulation values of Al, Ba, Cr, Cu, Fe, Pb, Sr and Zn related to *S. palustre* clone and *P. purum* exposed in bags for 6 weeks at ten Italian and Spanish sites. The values, expressed in ln(mg kg^{-1}) ± ln(SD) as error bars, are the averages, for each element, of the post- minus pre-exposure concentrations measured at all the exposure sites.

Table S1. Elemental concentrations (mean values; mg kg^{-1}) of *S. palustre* clone (C) in *Sphagnum* spp. from background world areas. n.d. = not detectable; < d.l. = under the detection limit. Colored cells indicate the lowest (blue) and the second lowest (light blue) concentration values. For the label codes see the text.

***Sphagnum* species**	**Geographical area**	**Al**	**As**	**B**	**Ba**	**Be**	**Cd**	**Ce**	**Co**	**Cr**	**Cs**	**Cu**	**Dy**	**Er**	**Eu**	**Fe**	**Ga**	**Gd**	**Hf**	**La**	**Lu**	**Mn**	**Mo**
C-U		4.9	0.021	6.9	0.06	0.0017	0.007	0.002	0.31	0.04	0.00039	0.79	0.00015	0.00010	0.00005	109	0.010	n.d.	0.001	0.002	≤ d.l.	109	3.1
C-100		56	0.018	13.3	0.02	0.0022	0.006	0.007	0.3	0.22	0.0026	0.79	0.00055	0.0002	0.00019	111	0.01	0.0008	0.0008	0.005	0.00006	103	2.95
C-EDTA		6.4	0.016	1.8	0.30	n.d.	0.004	0.001	0.010	0.0383	0.003	0.83	0.00015	0.00009	0.000059	80	0.007	0.0002	0.0005	0.001	≤ d.l.	6.5	3.0
C-EDTA100		1.8	0.015	2.0	0.28	n.d.	0.003	0.003	0.0096	0.07	0.0016	0.82	n.d.	0.0001	0.0001	93	0.0032	n.d.	0.00029	0.001	n.d.	6.2	2.8
S. palustre[1]	Poland		0.19		6.4				0.085		0.28					150				0.15			0.16
S. olafii[2] *	Greenland	322	0.114				0.041			0.705		1.03											0.041
S. cristatum[3]	New Zeland						0.0007					0.0039				2						0.0025	
S. girgensohnii[4]	Russia	254	0.11		17			0.38	0.37	0.26	0.31					297				0.19		114	
S. girgensohnii[5]	Russia	167	0.1		19	0.011	0.179	0.177	0.17	0.26	0.205		0.057	0.005	0.005	150	0.041		0.003	0.099	0.0006	341	0.126
S. girgensohnii[6]	Russia	254	0.11				0.18			0.25		2.1				297						113	
S. girgensohnii[7]	Finland															132							
S. girgensohnii[8]	Bulgaria	1043	0.67		56		0.3		0.33	1.7	0.28	7.6				864		0.23	0.078	0.6		173	
S. girgensohnii[9]	Russia	347	0.13		20		0.43	0.62	0.28	1.4	0.43					219			37	0.26		372	0.086
S. girgensohnii[9]	Bulgaria	1187	0.52		44		1.1	1	0.3	1.6	0.27					665			63	0.65		302	0.11
S. girgensohnii[10]	Russia	300			27	0.03	0.18	0.311	0.32	0.28		3.8	0.019	0.009	0.0058	330	0.07	0.07		0.16	0.0011	230	0.21
S. capillifolium[11]	Bulgaria	600					0.54		0.4			6.3				1200						198	
S. capillifolium[12]	Italy	1108	0.11				0.38		0.43	1.6		5.54				675						552	0.41
S. capillifolium[13]	Italy	1108	0.11				0.38		0.43	1.6		5.54				675						552	0.41
S. capillifolium[14]	Italy						0.9			1.37		4.3				506							
S. angustifolium[15]	Canada	537		4.8	233				0.2	1.1		8.2				383						10.5	0.5
S. auriculatum[16]	Portugal									0.2		7.5				156						0.22	
S. teres[11]	Bulgaria	500					0.48		0.2			5.1				600						246	
S. fuscum[17]	Canada																						
S. fuscum[18]	Sweden	318	0.25	2.98	13.69	0.008	0.22	0.93	0.29	0.70	0.14	4.11	0.037	0.035	0.015	349,00	0.65	0.14		0.60	0.004	102.8	0.21
S. papillosum[19]	Scotland																						
S. subsecundum[19]	Scotland																						
S. fallax[20]	Poland						0.25																
S. fallax[21]	Poland						0.16																
Sphagnum sp.[22]	Wales						2.9					3.8											
Sphagnum sp.[23]	England																						

Table S1. Continued.

Sphagnum species	Geographical area	Nb	Ni	Pb	Pr	Rb	Sb	Sm	Sn	Sr	Ta	Tb	Te	Th	Ti	Tl	Tm	U	V	W	Y	Yb	Zn
C-U		0.0008	0.11	0.016	0.0004	0.39	0.006	0.0004	0.02	1.7	0.00013	0.00004	n.d.	0.009	0.89	0.013	n.d.	0.003	0.046	0.006	0.0009	0.0002	52
C-100		0.0006	0.16	0.05	0.0009	0.38	0.017	0.0008	0.013	2.6	0.00009	0.00010	0.003	0.013	0.81	0.013	0.00005	0.0030	0.061	0.0047	0.0025	0.0003	51
C-EDTA		0.0005	0.06	0.02	0.00018	0.37	0.004	0.001	0.16	1.55	0.00009	≤ d.l.	n.d.	0.006	0.76	0.010	0.00005	0.003	0.05	0.004	0.0006	n.d.	8
C-EDTA100		0.0003	0.55	0.006	0.0002	0.36	0.06	0.0002	0.17	1.6	0.00008	≤ d.l.	n.d.	0.009	0.68	0.011	n.d.	0.0032	0.040	0.0037	0.0006	0.00016	9
S. palustre[1]	Poland					17	0.087	0.023						0.043									28
S. olafii[2] *	Greenland			0.16			0.002		0.013														5
S. cristatum[3]	New Zeland			0.0001																			0.023
S. girgensohnii[4]	Russia		2.5			71	0.04	0.021		7.6		n.d.		0.027	n.d.			0.015	0.5			n.d.	21
S. girgensohnii[5]	Russia	0.017		1.69	0.019	50.5			0.013	6.9	0.0009	0.0018	0.001	0.016	4.25	0.014	0.0007	0.007	0.52	0.027	0.053	0.045	23.7
S. girgensohnii[6]	Russia		2.4	2.2															0.54				20
S. girgensohnii[7]	Finland																						
S. girgensohnii[8]	Bulgaria		1.4			15	0.095	0.14		26	0.025	0.014		0.19	66			0.097	2.1				36
S. girgensohnii[9]	Russia		2.5			29	0.056	34		16	7			45				0.022	1	1			27
S. girgensohnii[9]	Bulgaria		2.1			12	0.085	88		41	21			140				0.057	3	0.52			60
S. girgensohnii[10]	Russia		1.6	3.5	0.035	65	0.04	0.03		10		0.034		0.019		0.022	0.0019		0.45			0.07	25
S. capillifolium[11]	Bulgaria		2.5	24																			35
S. capillifolium[12]	Italy		2.4	18.9											11.46				1.55				83
S. capillifolium[13]	Italy		2.4	18.9											11.46				1.55				83
S. capillifolium[14]	Italy		1.23	29.9															2.15				98
S. angustifolium[15]	Canada		0.8	0.1											22.3				1.3	1			46
S. auriculatum[16]	Portugal		3.1	30																			71
S. teres[11]	Bulgaria		2.3	19																			62
S. fuscum[17]	Canada			5.7																			20
S. fuscum[18]	Sweden		1.23	3.41	0.12	9.58	0.19	0.08		29.43		0.012		0.09		0.013	0.004	0.049	1.11		0.36	0.029	42.16
S. papillosum[19]	Scotland			19.6																			
S. subsecundum[19]	Scotland			20.1																			
S. fallax[20]	Poland			4																			27
S. fallax[21]	Poland			2.5																			
Sphagnum sp.[22]	Wales			4.9																			
Sphagnum sp.[23]	England			6.2																			

Underlined: mean concentrations of a sample constituted by both *S. fuscum* and *S. tenellum*. * mean concentrations of a sample constituted by both *S. olafii* and *Aulacomnium turgidum*.

References of Table S1

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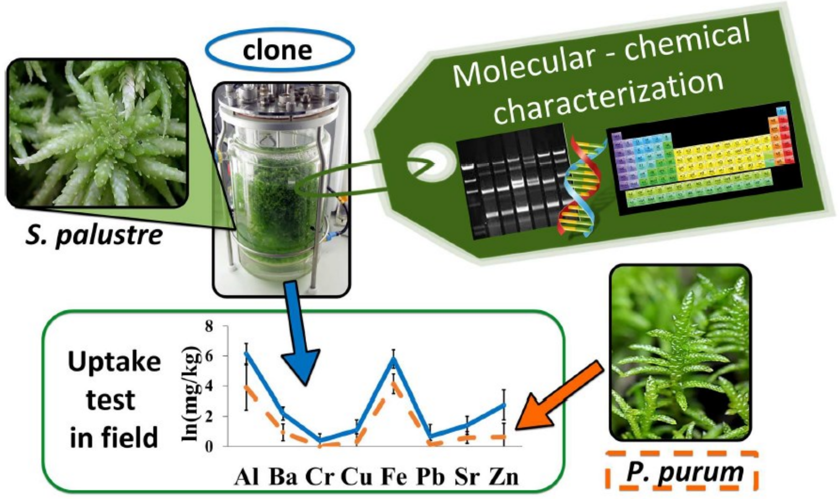
Graphical abstract



Chapter 5

Metal and proton adsorption capacities of natural and cloned *Sphagnum* mosses

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Abstract

Terrestrial mosses are commonly used as bioindicators of atmospheric pollution. However, there is a lack of standardization of the biomonitoring preparation technique and the efficiency of metal adsorption by various mosses. This is especially true for *in vitro*-cultivated moss clones, which are promising candidates for a standardized moss-bag technique. We studied the adsorption of copper and zinc on naturally grown *Sphagnum* peat moss in comparison with *in vitro*-cultivated *Sphagnum palustre* samples in order to provide their physico-chemical characterization and to test the possibility of using cultivated peat mosses as bioindicators of atmospheric pollution within the protocol of moss-bag technique. We demonstrate that *in vitro*-grown clones of *S. palustre* exhibit acid-base properties similar to those of naturally grown *Sphagnum* samples, whereas the zinc adsorption capacity of the clones is approx. twice higher than that of the samples from the field. At the same time, the field samples adsorbed 30-50% higher amount of Cu^{2+} compared to that of the clones. This contrast may be related to fine differences in the bulk chemical composition, specific surface area, morphological features, type and abundance of binding sites at the cell surfaces and in the aqueous solution of natural and cloned *Sphagnum*. The clones exhibited much lower concentration of most metal pollutants in their tissues relative to the natural samples thus making the former better indicators of low metal loading. Overall, *in vitro*-produced clones of *S. palustre* can be considered as an adequate, environmentally benign substitution for protected natural *Sphagnum* sp. samples to be used in moss-bags for atmospheric monitoring.

Keywords: *Biosorption, moss, Sphagnum, biomonitoring, moss-bag technique*

Highlights

- Natural and cloned mosses were compared for H and metal adsorption capacities.
- Metal adsorption capacity of mosses is related to DOC excreted by mosses.
- Cloned *S. palustre* has high specific surface area and metal adsorption capacities.
- *S. palustre* clones are optimal biosorbents for moss-bag technique.

1. Introduction

The use of terrestrial mosses as bioindicators of metal pollutant deposition from the atmosphere has been known since late sixties, when mosses began to be used for monitoring air pollution in Europe [1]. At that time, the scientific community understood that atmospheric pollution constituted one of the greatest challenges for the health of future human generations and for the environment [2-4]. Terrestrial mosses are more frequently used than other sorbents due to their lower cost, widespread presence and high adsorption capacity of pollutants [5]. One of the most commonly used methods for atmospheric biomonitoring is the “moss-bag technique” [6]. Native *Sphagnum* mosses are the most frequently used in the moss-bag technique, followed by *Pseudoscleropodium purum* and *Hypnum cupressiforme* [7]. Despite the broad application of this technique over the past decades, there is still a lack of (i) standardization of sample preparation for the moss-bag technique and (ii) justification of the choice of one particular moss species under variable environmental conditions.

The FP7 European project “MossClone” was focused on the *in vitro*-production of a moss clone and on the standardization of its application in moss-bags for the biomonitoring of air pollution by metals and polyaromatic hydrocarbons (PAHs) (http://www.mossclone.eu/). Having standardized the biomaterial and the procedure of exposure, data collected in different locations worldwide could be directly compared to assess the degree of air pollution. For this, it is crucial to conduct a physico-chemical characterization of the species collected from nature in comparison with *in vitro*-cultivated samples. A recent comparative study of several mosses demonstrated that *Sphagnum* sp. exhibits higher stability and metal adsorption capacities than *Pseudoscleropodium purum*, *Hypnum sp.* and *Brachythecium rutabulum* [8]. In the present study, *Sphagnum denticulatum* from the field and clonal, *in vitro*-produced *Sphagnum palustre* samples [9] were compared in terms of their adsorption capacity with respect to two important metal pollutants, and at the same time, essential micronutrients, Cu^{2+} and Zn^{2+}. These metals are also highly contrasting in terms of their affinity to organic ligands, water residence time in the hydration sphere and thus the rates of interaction with organic surfaces. It is thus anticipated that these metals encompass a wide range of physico-chemical properties of other divalent metal cations. *Sphagnum* species are considered as most relevant for the moss-bag technique due to their morphology [10], specifically the high number of pores in the hyalocysts, which help to trap airborne particulates [11]. However, a clear advantage of using *in vitro*-produced *Sphagnum* biomass is due to the fact that peat mosses and peatlands are often protected, e.g. via the European Council Habitat Directive (92/43/EEC) or the Fauna-Flora-Habitat Directive (92/43/EWG).

In the paper at hand, we report on the results of a quantitative comparison between *S. denticulatum* from the field and two clones of *S. palustre* produced under laboratory conditions [9]. Specifically, we aimed at testing whether *S. palustre* clones can be used as surrogate of natural *Sphagnum* mosses in terms of (i) surface area and acid-base properties; (ii) elemental chemical composition of the biomass, and (iii) divalent metal adsorption capacity from aqueous solutions.

2. Material and Methods

2.1. Chemicals

Copper and zinc nitrate (Sigma-Aldrich) were used as sources of Cu^{2+} and Zn^{2+}. The adsorption experiments were carried out in 0.01 M $NaNO_3$ and the solutions were prepared with Milli-Q water (18 MΩ). Buffer solutions were used in order to keep a constant pH during the adsorption. The MES (Merck) at pH 5.5 and HEPES at pH 6.5 buffers (Sigma-Aldrich) were used for the Cu^{2+} and Zn^{2+} experiments, respectively.

2.2 Moss samples

The clones of *S. palustre* were produced at the University of Freiburg (Germany) from samples collected in the south of Sweden [9]. Two randomly selected clones, called clone 2a and clone 12a, originated from two different spores. The plants were grown in 5 L photobioreactors with liquid Knop medium [12] supplemented with micronutrients, sucrose and ammonium nitrate [9]. Because peat-forming natural *S. palustre* is protected in Europe (92/43/EEC, 92/43EWG), natural specie, *S. denticulatum,* was collected in 2012 in NW Spain in a non-urban area. The samples, consisted of mostly apical, green parts, were rinsed 3 times in Milli-Q water and oven-dried at 120 °C.

2.3 Moss characterization

The elemental composition of peat mosses was measured after full acid digestion via microwave (MARS 5 system CEM) treatment in the A 10,000 Clean Laboratory Facilities of GET (CNRS-Toulouse). Full acid digestion was carried out on 100 mg of dried plant material with bidistilled HNO_3 (9 mL), suprapure HF (0.2 mL; Merck) and suprapure H_2O_2 (1 mL; Merck) in 20 mL Teflon containers. The digestion conditions were as follows: 1600 W for 8 min, keeping the temperature at 150 ˚C for 20 min. The standard used was BCR-482 lichen (Certified Reference Material, IRMM). The final solution (after digestion) was evaporated at 60 ˚C in a hot plate and re-diluted in 2% HNO_3. All major and trace elements except Si were measured by ICP-MS (Agilent 7500 ce series) with a detection limit of 0.001-0.0003 $\mu g\ g^{-1}$ and precision of ±5%. Further details of moss analyses are given in Ref. [13].

The specific surface area (SSA) of peat moss samples was measured using the B.E.T. N_2 multipoint adsorption technique using the Quantachrome Autosorb Automated Gas Sorption System with 10 to 14 adsorption points after 24 h of degasing at 120 °C. The typical uncertainty of these measurements was 10%.

The surface acid-base titration was conducted in 0.01 M $NaNO_3$ at room temperature (20 ± 1 ºC). Aqueous suspension of 1 $g_{dw}\ L^{-1}$ was conditioned for 1 h and pre-saturated with N_2 at pH from 3 to 11. Acidic and basic titration was performed in two steps by adding aliquots of 0.1 M HCl and 0.1 M NaOH, respectively. All titration experiments were performed in triplicates. The reference solution was a biomass-free supernatant solution after 1 h conditioning under N_2 bubbling. The pH was measured by a combined electrode (Mettler Toledo^R) in a pH-meter ion analyzer (pHM250-Meterlab™) with an uncertainty of ± 0.002 units. The excess of charge was computed as the difference of the acid/base concentration in the suspension and in the reference solution according to common procedures of biomass titration [14,15]. Dissolved Organic Carbon (DOC) leached from the moss biomass during the experiments was monitored using a Carbon Total Analyzer (Shimadzu TOC-V_{CSN}) with an uncertainty of 2% and a detection limit of 0.1 mg L^{-1}, as a function of pH, at experimental conditions similar to those of the titration and metal adsorption.

2.4 Metal adsorption experiments

The peat mosses were rinsed 3 times with Milli-Q (18 MΩ) and devitalized at 120˚C overnight [16]. Oven treatment allows devitalization of the biomass, thus eliminating the metabolic influence on the adsorption or uptake of metals, reducing the data variability [17-20]. Intact tissue was always used without grinding or disaggregation in order to meet the typical conditions of the moss-bag technique. The biomass was kept constant at 1 $g_{dry}\ L^{-1}$ for all the adsorption experiments.

The adsorption experiments were carried out following the protocol elaborated for field samples of mosses [8]. The adsorption of Cu^{2+} and Zn^{2+} was investigated in 0.01 M $NaNO_3$ as a function of pH and total metal concentration in solution. The aqueous solution was undersaturated with respect to the metal oxide or carbonate as verified by speciation calculations with the Visual Minteq computer code [21]. The experiments were conducted in polypropylene beakers at room temperature (20 ± 1°C) and agitated with a suspended Teflon coated magnetic stirrer under continuous nitrogen bubbling.

The pH-edge adsorption experiments were performed at a constant initial concentration of Cu^{2+} (52 µmol L^{-1}) and Zn^{2+} (51 µmol L^{-1}). The pH ranged from 1.8 to 6.7 and from 1.8 to 9.9 for the Cu^{2+} and Zn^{2+} experiments, respectively. The pH was adjusted by adding aliquots of NaOH (0.1 – 0.01 M) or HNO_3 (0.1 – 0.01 M). The adsorption of metals as a function of their concentration in solution was carried out in the range of 5 to 3624 µmol L^{-1} for Cu^{2+} and 4 to 3500 µmol L^{-1} for Zn^{2+}. The pH was kept constant at ~ 5.5 and ~ 6.5 for Cu^{2+} and Zn^{2+}, respectively. The elapsed time between each metal addition or the pH change was always 10 min, sufficient for establishing the adsorption equilibrium according to previous kinetic experiments [8]. The control solution for adsorption experiments was 0.45 µm-filtered moss leachate produced by interaction of the moss biomass with metal-free inert electrolyte during 1 h.

Sampled solution was filtered (0.45 µm, acetate cellulose single used filter), acidified with bidistilled HNO_3, and analyzed for aqueous metal concentration using flame atomic adsorption spectroscopy (Perkin Elmer AAnalyst 400) with an uncertainty of ± 2% and a detection limit of 0.05 mg L^{-1}.

The results of the acid-base titration and metal adsorption experiments were treated with a Linear Programming Model (LPM) in order to compute the apparent equilibrium constants and the site densities for each individual experiment following the approaches elaborated for bacteria [14,15,22-25]. This model is highly appropriate for describing complex 3-D multi-layer systems with both organic components and rigid cell walls [23,26,27] and is capable of adequately describing proton and metal adsorption on natural mosses [8].

To account for Cu^{2+} and Zn^{2+} complexation with organic ligands leached from the biomass, we used the NICA-Donnan model and Soil Humic Model (SHM) for natural organic complexation with metals (see Ref. [28] for application in aqueous solutions) implemented to vMinteq (version 3.1, Gustafsson [21]).

3. Results

3.1 Specific surface area and elemental composition of the analyzed peat mosses

The clones exhibited a higher B.E.T. surface area (clone 2a = 24 ± 1 m^2 g_{dry}^{-1}; clone 12a = 28 ± 1 m^2 g_{dry}^{-1}) compared to natural samples (10.8 ± 0.3 m^2 g_{dry}^{-1}), as determined by 10 to 14 points adsorption isotherm, with a correlation coefficient that was always higher than 0.99. The differences between clones and natural samples were statistically significant ($p < 0.05$). We found that the reliable SSA measurements require degassing at 120°C overnight. Heating in vacuum at 80°C did not yield sufficient degassing and either N_2 adsorption isotherm could not be produced or significant (> 20%) underestimation of $SSA_{B.E.T.}$ occurred.

Elemental composition is one of the key factors controlling the adsorption capacity of different biosorbents, notably at low metal concentration in the environment. These results are listed in Table 1. For comparison, metal content of *S. denticulatum* from the field [8] and the average of 11 *Sphagnum* species from pristine western Siberian habitats [13] are also listed. The clones reflect the composition of the culture medium because the strains were not exposed to any atmospheric pollution. Among ~ 40 major and trace elements, only nutrients P, K and Mo were slightly higher in the clones compared to the samples from nature. All other major and trace elements were significantly higher in natural *Sphagnum*, reflecting metal deposition from the atmosphere in their original location. Thus, *S. denticulatum* contained 3.55 mg kg^{-1} and 36.0 mg kg^{-1} of Cu and Zn, respectively, which is higher than both clones by a factor of 2 to 4. The other major and trace metals were lower by a factor of 2 to > 10 in laboratory clones compared to field samples of *S. denticulatum.* The impoverishment of clones versus natural samples was especially remarkable for Al (x 300), V (x 400), Ga (x 100), As (x 500), Cd (> 1000), REEs (> 1000) and Pb (x 400).

3.2 Acid-base titration

The results of the surface acid-base titration carried out at pH 3 to 10 in triplicates for 120°C-devitalized mosses are depicted in Figure 1. The pH value corresponding to zero net proton adsorption (pH_{PZC}) was equal to 4.5 ± 0.1 for the peat moss sample from the field, which was significantly lower than that of the clones (clone 2a: 5.65 ± 0.05, clone 12a: 5.60 ± 0.06). The excess of charge was similar for both clones, and the magnitude of the surface charge of the clones was significantly higher than that of the field sample.

The experimental data of H^+/OH^- adsorption were fitted by LPM to calculate the acidity constants which could be linked to functional groups on the surface of the cells (Table 2). All analyzed peat mosses contained five possible functional groups: carboxyl/phosphodiester, carboxyl, phosphoryl, amine and polyphenols with total binding sites of 0.648 mmol g_{dry}^{-1} (field sample), 1.56 mmol g_{dry}^{-1} (clone 2a) and 1.31 mmol g_{dry}^{-1} (clone 12a). The two clones had 1.04 and 0.742 mmol g_{dry}^{-1} of carboxyl/phosphodiester ($pK_a = 3.3 – 5.8$) respectively, which was higher by a factor of 2 to 3 than that of *S. denticulatum*, having only 0.355 mmol g_{dry}^{-1}. In terms of the phosphoryl groups, clone 12a exhibited one order of magnitude higher value than that of clone 2a (0.817 and 0.079 mmol g_{dry}^{-1}, respectively) and the field sample (0.043 mmol g_{dry}^{-1}). The clones also exhibited a 1.5 times higher concentration of surface amines (0.16 – 0.18 mmol g_{dry}^{-1}, $pK_a = 8.05 – 9.35$) and polyphenols (0.29 – 0.24 mmol g_{dry}^{-1}, $pK_a \sim 10.30$) relative to natural sample (0.09 and 0.16 mmol g_{dry}^{-1}, respectively). Consequently, the peat moss grown under natural conditions demonstrated a 1.5 to 2 times lower mass-normalized binding site density compared to the clones, and thus their metal adsorption capacities should also be lower than those of clones.

3.3 Adsorption of Cu2+ and Zn2+ as a function of pH

Results of adsorption of Cu^{2+} and Zn^{2+} onto peat mosses as a function of solution pH are plotted in Figure 2 and the LPM adsorption parameters are listed in Table 3. The adsorption of Cu^{2+} achieved the maximum at pH 6.2 – 6.7 and was 73 – 75% of the initial amount for the clones and 82% for the field samples. The 50% removal of initially added metal occurred at pH 4.4 and 4.0 – 4.1 for both field sample and *in vitro*-cultivated clones. In the case of Zn^{2+}, the maximum adsorption was achieved at pH 7.3 – 7.6 and ranged from 72% (field sample) to 82% (clone 12a). The 50% of initially loaded Zn^{2+} was adsorbed at pH 5.2, 5.4 and 3.6 for the field sample, clone 2a and clone 12a respectively.

The analyses of obtained LPM parameters including the equilibrium constant (pK_s) and available binding sites demonstrated that there are significant differences in Cu^{2+} adsorption parameters between *Sphagnum* moss from nature and clones from *in vitro* culture: natural *Sphagnum* had two pK_s values (-2.20 and -0.15), while clone 2a exhibited three pK_s (-1.25, -0.15 and 2.60), and clone 12a had four pK_s (-1.45, -0.45, -0.20 and 3.0). Note that, within the LPM formalism, the lower the pKs value, the stronger the interaction between the metal and the binding site. In this study, the lowest pK_s, at -2.20, was achieved for the field samples. The total concentration of Cu^{2+}-binding sites was slightly higher in clones 2a and 12a (48 and 49 µmol g_{dry}^{-1}, respectively) compared to natural *Sphagnum* (43 µmol g_{dry}^{-1}).

The equilibrium constants for the pH edge of Zn^{2+} adsorption ranged from -1.55 to 1.65. Both *S. palustre* clones presented a slightly higher concentration of binding sites relative to the field sample: 32, 37 and 43 µmol g_{dry}^{-1} for the field sample, clone 2a and clone 12a respectively. Clone 12a exhibited the lowest value of pK_s (-1.55) and the highest level of binding sites (42.5 µmol g_{dry}^{-1}).

The DOC concentration in pH-dependent adsorption experiments with these three studied peat moss samples is shown in Figure 3. Natural *S. denticulatum* excreted a factor of 3 to 4 lower amount of DOC compared to the clones of *Sphagnum palustre*. Namely, peat moss from the field produced 14.5 mg L^{-1} to 20.5 mg L^{-1} of DOC from pH 1.7 to 10.9. For the same pH range (3.4 to 9.4), clone 2a excreted a slightly higher amount of DOC than clone 12a (41 mg L^{-1} to 49 mg L^{-1}, and 28 mg L^{-1} to 37 mg L^{-1}, respectively).

3.4 Adsorption of Cu2+ and Zn2+ as a function of metal concentration in solution

The results of Cu^{2+} and Zn^{2+} adsorption as a function of these metal concentrations in solution are shown in Figure 4. The LPM fitting parameters are listed in Table 3. The "Langmuirian" adsorption isotherms were obtained at constant pH of ~5.5 and ~6.5 for Cu^{2+} and Zn^{2+}, respectively, and the metal concentration ranged from 4 to 3920 µmol L^{-1}. The field sample demonstrated an approx. 30% higher adsorption capacity of Cu^{2+} in comparison with *in vitro*-cultivated *S. palustre*. Although the total binding site concentration was equal to 1.07 mmol g_{dry}^{-1}, 24.0 mmol g_{dry}^{-1} and 1.90 mmol g_{dry}^{-1} for field samples, clone 2a and 12a respectively, the stabilities of these sites for the clones were lower, as reflected in their pK_m values of Cu adsorption (Table 3). In the case of Zn^{2+}, the clones demonstrated a factor of 2 higher adsorption capacities relative to the field samples. The total number of Zn-binding sites was equal to 94.1 mmol g_{dry}^{-1}, 157 mmol g_{dry}^{-1} and 750 mmol g_{dry}^{-1} for the field sample, clone 2a and clone 12a respectively.

In addition to the LPM, experiments were fitted by a Langmuirian adsorption isotherm, often used for description of metal adsorption on biosorbents [29]. Here, it allowed rationalizing the adsorption data according to:

$$\frac{[Me^{2+}]_{aq}}{[Me^{2+}]_{ads}} = \frac{1}{K_L q_{\max}} + \frac{[Me^{2+}]_{aq}}{q_{\max}} \qquad (1)$$

where K_L is the Langmuir equilibrium (g_{dry} $mmol^{-1}$) constant and q_{max} is the maximum adsorption capacity (mmol g_{dry}^{-1}). All the experiments were adequately fitted by Equation 1 with $R^2 > 0.98$; the results are listed in Table 4. The maximum adsorption capacity of copper was achieved for the field sample (1.25 mmol g_{dry}^{-1}), and that of Zn^{2+} was a factor of 4 higher for clones compared to the field sample.

The results of thermodynamic modeling of Cu and Zn complexation with DOM leached from peat mosses at pH of 5.4 ± 0.1 and 6.5 ± 0.1 for Cu and Zn, respectively, are shown in Figure 5. It can be seen that at typical metal concentration in solution (0.4 mmol L^{-1}) the difference in Cu-free concentration between 15 and 45 ppm DOM solution is a factor of 1.7 while for Zn this factor is 1.4. At lower concentration of Cu, > 99% of this metal may be complexed with aqueous organic ligands, whereas for Zn this amount never exceeds 84%. In other words, at otherwise similar < 0.45 µm metal concentration, the concentration of free Zn^{2+} available for adsorption onto clones is more than an order of magnitude higher than that of free Cu^{2+}. Note that the modeling of Cu and Zn complexation within SHM formalism yielded a very similar degree of complexation with DOM as a function of metal concentration. However, it is not ruled out that the organic complexes of metals can also be adsorbed on the moss surfaces which make rigorous modeling of aqueous and surface complexation very difficult at the present time.

4. Discussion

The B.E.T. measurements demonstrated that *in vitro*-grown clones had a factor of 2 higher specific surface areas than the field samples. According to morphological observations, shoots of *S. palustre* from the field are thicker and heavier than of those grown *in vitro* [9]. The average dry weight of field shoots (n=10) was 14.76 ± 7.2 mg, while the *in vitro*-cultivated material had an average shoot (n=13) weight of 2.72 ± 1.13 mg. Another rather variable morphological trait was the number of pores per hyalocyste: 3 to 16 pores for native moss and 2-3 pores for the clones of *S. palustre* [9]. However, in virtue of a high variability, the clones showed significantly lower and constant number of pores per hyalocyste to native ones. These results confirm that cloned species are less variable in surface morphology than natural samples. Therefore, cloned peat mosses are highly beneficial for the biomonitoring relative to the natural samples.

The SSA measured for *S. denticulatum* from the field (10.8 m^2 g_{dry}^{-1}) is significantly higher than the geometric surface area of the moss *H. cupressiforme* (0.136 m^2 g^{-1}) and the lichen *Pseudevernia furfuracea* (0.027 m^2 g^{-1}), Ref. [17], while it is comparable with SSA of Danish peat (13.3 m^2 g^{-1}) and Heilongjiang peat (9.67 m^2 g^{-1}), Ref. [30]. In terms of acidity constants, the functional groups were carboxyl/phosphodiester, carboxyl, phosphoryl, amine and polyphenols, corresponding to pKa of ~3.30 to 3.50, 4.85–5.85, 6.10–7.35, 8.05–9.35 and 10.30–10.35, respectively. Note that the most relevant functional groups for metal binding on peat mosses are carboxylates and phenolates [31]. Natural *S. denticulatum* had ~55% less mass-normalized total binding sites compared to clones. This difference disappears when normalizing the site number to the $SSA_{B.E.T.}$, which is a factor of 2 higher for clones compared to the field sample. As such, proton-binding properties of clone surfaces can be considered very similar to those of natural *Sphagnum*.

Langmuirian isotherms suggested that the field sample is able to adsorb 46 to 13% higher amounts of Cu^{2+} (q_{max} = 1.25 mmol g_{dry}^{-1}) compared to clones (q_{max} = 0.67 and 1.09 mmol g_{dry}^{-1} for clone-2a and 12a, respectively). However, the capacity of clones to adsorb Zn^{2+} is 76-80% higher (q_{max} = 3.87 and 4.60 mmol g_{dry}^{-1} for clones 2a and 12a, respectively) than that of *S. denticulatum* (q_{max} = 0.93 mmol g_{dry}^{-1}).

The Langmuirian equation, valid at specific and constant pH of aqueous solution, assumes that (1) adsorption is limited to the formation of a monolayer and (2) all surface sites have the same energy or equal affinity for metal [32,33]. The LPM can be considered as multi-langmuirian model with each site having its own adsorption energy and affinity [22]. In this regard, two approaches are not quantitatively comparable, and the sum of all binding sites in the LPM (Table 3, constant-pH isotherm) is always higher than the qmax value of Eq. (1) (Table 4). In fact, the contribution of each site in the LPM may be pronounced at very narrow pH or [Me^{2+}] concentration, since their complexation constants differ by 3–4 orders of magnitude. Therefore, the sum or the average of multiple LPM parameters cannot be compared with one single site number and stability constant of the langmuirian formalism. Note however, that the results of the present study are consistent with previous evaluations of langmuirian adsorption parameters of biological surfaces, as compiled in Refs. [34,35]. For example, the values of qmax of this study are also comparable with those reported for *P. purum*, *Hypnum* sp. and *B. rutabulum* [8] and higher than that of aquatic plants [36–38], fungi [39,40], bacteria [41,42] and land plants [43].

The solution pH is one of the most important parameters affecting the biosorption of heavy metal ions [44, 45]. Increasing the pH in solution leads to deprotonation of the available sites on peat moss surface layers, which thus increases the adsorption capacity with the pH rise. The adsorption of Cu^{2+} and Zn^{2+} followed the "universal pH-edge adsorption pattern" already identified for mosses [8], cyanobacteria [25], phototrophic bacteria [15], heterotrophic bacteria [46-48] and diatoms [49]. The most significant Zn^{2+} adsorption capacity was found for clone 12a, when 50% of Zn^{2+} was adsorbed at pH 3.6, which are 2 pH units lower than that for clone 2a and the samples from nature. The clone 12a also exhibited the lowest pK_s value (-1.55) and the highest amount of total binding sites (49.0 μmol g_{dry}^{-1}).

The production of DOM can affect the speciation of metals in solution and thus interfere in the adsorption process via decreasing the activity of free metal available for adsorption [50]. The nature of this DOM cannot be characterized unambiguously but presumably comprise cell exometabolites and nutrient organic compounds trapped in the surface pores. Given that the $SSA_{B.E.T.}$ of clones is a factor of 2 higher than that of native *Sphagnum*, the retaining DOM capacity of clones is significantly higher. Copper is known to be strongly bound by organic ligands excreted by organisms [51]. These authors compiled the following order of the metal affinity complexation by natural organic ligands: $Cu^{2+} > Ni^{2+} > Zn^{2+} > Co^{2+} > Cd^{2+} > Ca^{2+} > Mg^{2+}$, with the conditional stability constant of Cu^{2+} complexes being several orders of magnitude higher than that of Zn^{2+} (log K of 13.5 to 16 and 7.8 to 9.5, respectively). Hirose [52] also reported a much stronger affinity of Cu^{2+} to organic ligands compared to that of Zn^{2+}. Moreover, Smith, Bell and Kramer [53] compiled the data from 13 carboxylic, 4 amine, 19 amino-carboxylic and 5 sulfidic-thiolate ligand compounds and demonstrated that the stability constant was significantly higher for Cu^{2+} than for Zn^{2+}.

The concentration of DOC excreted by the studied peat mosses (Fig. 3) thus may explain the difference in the adsorption capacity with respect to the two studied metals (Fig. 4A). The speciation calculation demonstrated that Cu^{2+} was significantly more bound to organic ligands produced by peat moss reaction with aqueous solution than Zn^{2+}. For this reason, only small fraction of total dissolved Cu^{2+} was present in free forms, available for adsorption (Fig. 5). This amount, however, was much higher for natural sample versus clone due to 2-3 times lower DOC concentration in the former (Fig. 3). As such, natural *Sphagnum* could adsorb more Cu^{2+} from solution than the clones. Based on the results of these calculations, and considering the divalent metal affinity to organic ligands [51], we expect that the majority of other metals (Co, Cd, Pb, alkaline-earths) will follow Zn rather than Cu and will be adsorbed more strongly by clones compared to *Sphagnum* collected from the field.

In addition to carboxylate groups, representing the major part of proton and metal binding sites, more specific and metal-selective moieties like phosphoryl, amine, and sulfhydryl also play important roles in the adsorption of metals. However, these groups could be screened (protected) by mineral coating formed during lifetime of natural samples. Therefore, an additional explanation for the lower adsorption capacity of naturally grown peat moss compared to clones is the significantly higher Fe and Al concentration in the field samples (Table 1). Fe and Al hydroxides and clay aluminosilicates, deposited from the atmosphere, are capable of blocking, as a protective layer, the active binding sites and decrease the concentration of specific organic moieties controlling metal complexation at the surface. In fact, thin hydroxide/clay coating, although being an efficient sorbent, may not allow protons and metals to penetrate within the moss surface layer, where they can interact with multiple surface sites, as it is known for other biological surfaces [49,54]. However, given the lack of direct evidence on hydroxide coating on mosses, this assumption remains to be verified by surface-sensitive techniques such as X-ray Electron Spectroscopy (e.g., Ref. [55]).

It follows that the use of cloned moss samples instead of natural species for passive biomonitoring and atmospheric pollution assessment has double benefit. First, clones exhibit a lower variability in both chemical and physical properties, allowing highly homogeneous stock of virtually identical biosorbents. Second, the concentrations of metal pollutants, metalloids and trace elements in cloned moss biomass are significantly lower than those in native samples, thus enabling efficient use of the clones during short periods of exposure to capture short-term pollution events

5. Conclusions

This study provides physico-chemical background for unifying the method of atmospheric metal biomonitoring using the mossbag technique. For this, we quantified the metal and proton adsorption of *in vitro*-cultivated *Sphagnum* moss as well as the species collected from the field, as a function of pH and Cu2+ and Zn2+ concentration. We demonstrated that *S. palustre* clones exhibit prominent physico-chemical characteristics to be used instead of environmentally-protected natural Sphagnum mosses. In particular, clones have a larger specific surface area and greater mass-normalized adsorption capacities compared to field samples, especially for Zn^{2+} and presumably for many other divalent metals. These differences in metal and proton adsorption between clones of *S. palustre* and

S. denticulatum can be understood in terms of the composition of the cell walls, type and abundance of ligands in the cell walls, and the morphological features of field samples and in vitro-cloned samples. Laboratory clones contain a factor of 10–1000 lower concentrations of many atmospheric pollutants, compared to natural samples, and as such they can be used even for short period of biomonitoring in low-contaminated environments. Further work is necessary in order to reveal the mechanisms of surface and bulk enrichment in metals of natural mosses compared to cloned samples. Overall, the in-vitro production of *S. palustre* clones can provide an optimal biosorbent material for monitoring atmospheric metal deposition using the standardized moss-bag technique.

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Tables

Table 1. Major and trace elemental composition of natural *Sphagnum denticulatum* (collected from the field as described in Gonzalez and Pokrovsky, 2014), natural *Sphagnum* sp. collected in pristine regions of western Siberia across a 1800 km profile (average of 11 sites with 2 s.d., Stepanova et al., 2015) and laboratory clones *S. palustre* (2a and 12a). The detection limit is approx. 0.0003 mg kg^{-1}. N.D. stands for non-determined.

[Me] mg kg^{-1}				
Element	**Siberian *Sphagnum***	**Field samples**	**Clone 2A**	**Clone 12A**
Be	N.D.	0.23	< 0.05	< 0.05
B	7.0±4.3	0.471	0.576	0.557
Na	337±155	525	0.726	N.D.
Mg	572±183	1630	378	387
Al	1336±1143	1048	2.30	2.56
P	360±133	390	502	545
K	1917±1265	2595	1151	1567
Ca	1696±749	3365	846	906
Ti	89±72	16.5	0.611	0.744
V	2.4±2.0	1.21	0.003	0.002
Cr	2.2±1.4	0.460	0.282	0.107
Mn	82.7±73	162	30.6	33.1
Fe	1224±908	1146	150	141
Co	0.51±0.32	0.238	0.034	0.034
Ni	1.77±1.0	0.935	0.176	0.085
Cu	2.66±1.4	3.55	0.803	0.605
Zn	27.9±11.1	36.0	18.1	19.0
Ga	0.41±0.3	0.232	0.002	0.003
Ge	N.D.	0.059	< 0.01	< 0.01
As	1.11±0.4	0.524	0.001	< 0.001
Rb	6.69±4.25	10.22	0.047	0.063
Sr	11.2±9.4	43.79	0.334	0.348
Zr	2.3±1.8	0.287	0.039	0.039
Nb	N.D.	0.105	0.003	0.002
Mo	0.24±0.2	0.204	1.567	1.853
Ag	N.D.	0.011	0.002	0.002
Cd	0.43±0.2	0.203	< 0.001	< 0.001
Cs	0.23±0.1	0.228	< 0.002	< 0.002
Ba	22.7±9.4	44.7	0.047	0.048
La	0.56±0.4	32.1	0.011	0.010
Ce	1.16±0.93	6.83	0.013	0.011
Pr	0.13±0.1	3.357	0.002	0.002
Nd	0.51±0.4	12.6	0.010	0.008
Sm	0.10±0.08	1.68	0.002	0.001
Eu	0.02±0.02	0.168	< 0.0003	< 0.0003
Gd	0.09±0.07	1.65	0.002	0.001
Dy	0.081	0.833	0.001	0.001
Ho	0.014	0.144	< 0.0003	< 0.0003
Er	0.043	0.371	0.001	0.001
Tm	0.007	0.039	< 0.0003	< 0.0003
Yb	0.04	0.245	< 0.0003	< 0.0003
Lu	0.007	0.026	< 0.0003	< 0.0003
Hf	0.16±0.15	0.019	0.002	0.001
Ta	N.D.	0.019	0.001	≤ d.l.
Pb	8.6±4.7	42.9	0.129	0.057
Th	0.08±0.06	0.033	< 0.0003	< 0.0003
U	0.07±0.06	0.043	< 0.0003	< 0.0003

Table 2. Surface acid-base titration and LPM model results for *S. denticulatum* and *S. palustre* clones produced in the laboratory reactors. The experiments were performed in 0.01 M $NaNO_3$ electrolyte solution with a constant biomass of 1 g_{dry} L^{-1} in triplicates.

Species	**pK_a**	**Binding sites (mmol g_{dry}^{-1})**	**Functional Groups**
Sphagnum denticulatum (field sample)	3.50	0.205	Carboxyl - Phosphodiester
	4.85	0.108	Carboxyl
	5.85	0.042	Carboxyl
	6.55	0.020	Phosphoryl
	7.20	0.023	Phosphoryl
	8.05	0.028	Amine
	9.15	0.066	Amine
	10.30	0.156	Polyphenol
Sphagnum palustre (clone 2a)	3.30	0.920	Carboxyl - Phosphodiester
	5.80	0.120	Carboxyl
	7.35	0.079	Phosphoryl
	8.10	0.006	Amine
	9.35	0.154	Amine
	10.30	0.285	Polyphenol
Sphagnum palustre (clone 12a)	3.30	0.742	Carboxyl - Phosphodiester
	6.10	0.743	Phosphoryl
	7.25	0.074	Phosphoryl
	8.10	0.021	Amine
	9.05	0.161	Amine
	10.35	0.237	Polyphenol

Table 3. List of experiments performed in this study, experimental conditions and LPM parameters. All metal adsorption experiments were performed in 0.01 M $NaNO_3$ with a constant biomass 1 g_{dry} L^{-1}. K_s and K_m correspond with the equilibrium constant for the reaction between metal in solution and the available sites as a function of pH and metal aqueous concentration in solution, respectively.

Metal	**Type of study**	**Species**	**pH-range**	**$[Me^{2+}]_0$ µM**	**pK_s/pK_m**	**Binding Sites (mol g_{dry}^{-1})**
Cu	pH-edge	*Sphagnum denticulatum* (field sample)	1.8 – 6.5	51.94	-2.20	$1.03\bullet10^{-5}$
					-0.15	$3.29\bullet10^{-5}$
		Sphagnum palustre (Clone-2a)	2.1 - 6.7		-1.25	$2.80\bullet10^{-5}$
					-0.15	$1.27\bullet10^{-5}$
					2.60	$7.66\bullet10^{-6}$
		Sphagnum palustre (Clone-12a)	2.1 - 6.7		-1.45	$2.07\bullet10^{-5}$
					-0.45	$1.39\bullet10^{-5}$
					-0.20	$2.60\bullet10^{-6}$
					3.0	$1.18\bullet10^{-5}$
Zn		*Sphagnum denticulatum* (field sample)	1.8 – 9.7	50.48	-0.80	$1.44\bullet10^{-5}$
					0.55	$1.79\bullet10^{-5}$
		Sphagnum palustre (Clone-2a)	1.9 - 9.9		-0.8	$8.09\bullet10^{-6}$
					-0.35	$8.98\bullet10^{-6}$
					0.95	$2.10\bullet10^{-6}$
		Sphagnum palustre (Clone-12a)	2.1 - 9.6		-1.55	$3.28\bullet10^{-5}$
					0.35	$5.52\bullet10^{-6}$
					1.65	$4.15\bullet10^{-6}$
Cu	Constant pH isotherm	*Sphagnum denticulatum* (field sample)	5.53 ± 0.03	5 - 3624	0.50	$2.33\bullet10^{-5}$
					2.45	$1.05\bullet10^{-3}$
		Sphagnum palustre (Clone-2a)	5.4 ± 0.1	4 - 3920	0.6	$4.39\bullet10^{-6}$
					2.05	$4.57\bullet10^{-4}$
					5.30	$2.35\bullet10^{-2}$
		Sphagnum palustre (Clone-12a)	5.4 ± 0.1	4 - 3838	1.60	$2.03\bullet10^{-4}$
					3.25	$1.67\bullet10^{-3}$
Zn		*Sphagnum denticulatum* (field sample)	6.7 ± 0.1	7 - 3500	2.75	$2.16\bullet10^{-4}$
					6.30	$9.39\bullet10^{-2}$
		Sphagnum palustre (Clone-2a)	6.48 - 0.04	4 - 3145	1.80	$6.23\bullet10^{-5}$
					3.60	$1.33\bullet10^{-4}$
					6.10	0.157
		Sphagnum palustre (Clone-12a)	6.42 - 0.05	4 - 3037	2.00	$1.02\bullet10^{-4}$
					6.70	0.747

Table 4. Langmuir parameters computed from the experiments at different aqueous metal concentrations listed in Table 3 and plotted in Figure 3 (Langmuirian isotherm).

Species	q_{max} (mmol g_{dry}^{-1})	K_L (g_{dry} mmol^{-1})
Copper		
Sphagnum denticulatum (field)	1.25	0.47
Sphagnum palustre (clone 2a)	0.67	0.78
Sphagnum palustre (clone 12a)	1.09	0.39
Zinc		
Sphagnum denticulatum (field)	0.93	0.44
Sphagnum palustre (clone 2a)	3.87	0.11
Sphagnum palustre (clone 12a)	4.60	0.09

Figures

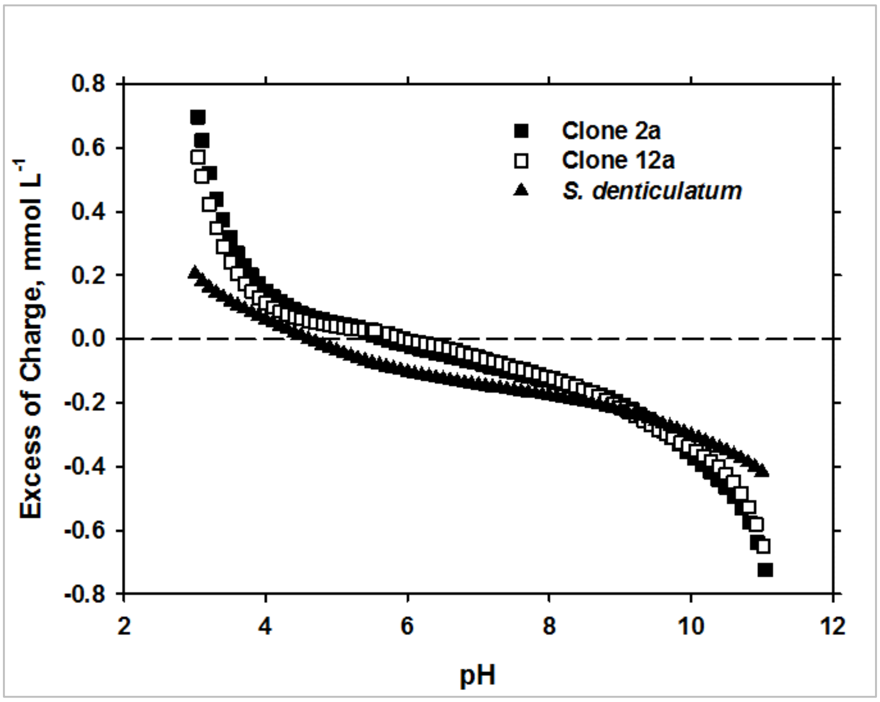


Figure 1. Surface acid-base titration of *S. denticulatum* and *S. palustre* clones in 0.01 M $NaNO_3$. The conditioning time was equal to 1h and the biomass was kept constant at 1 g_{dry} L^{-1}. All the measurements were triplicated.

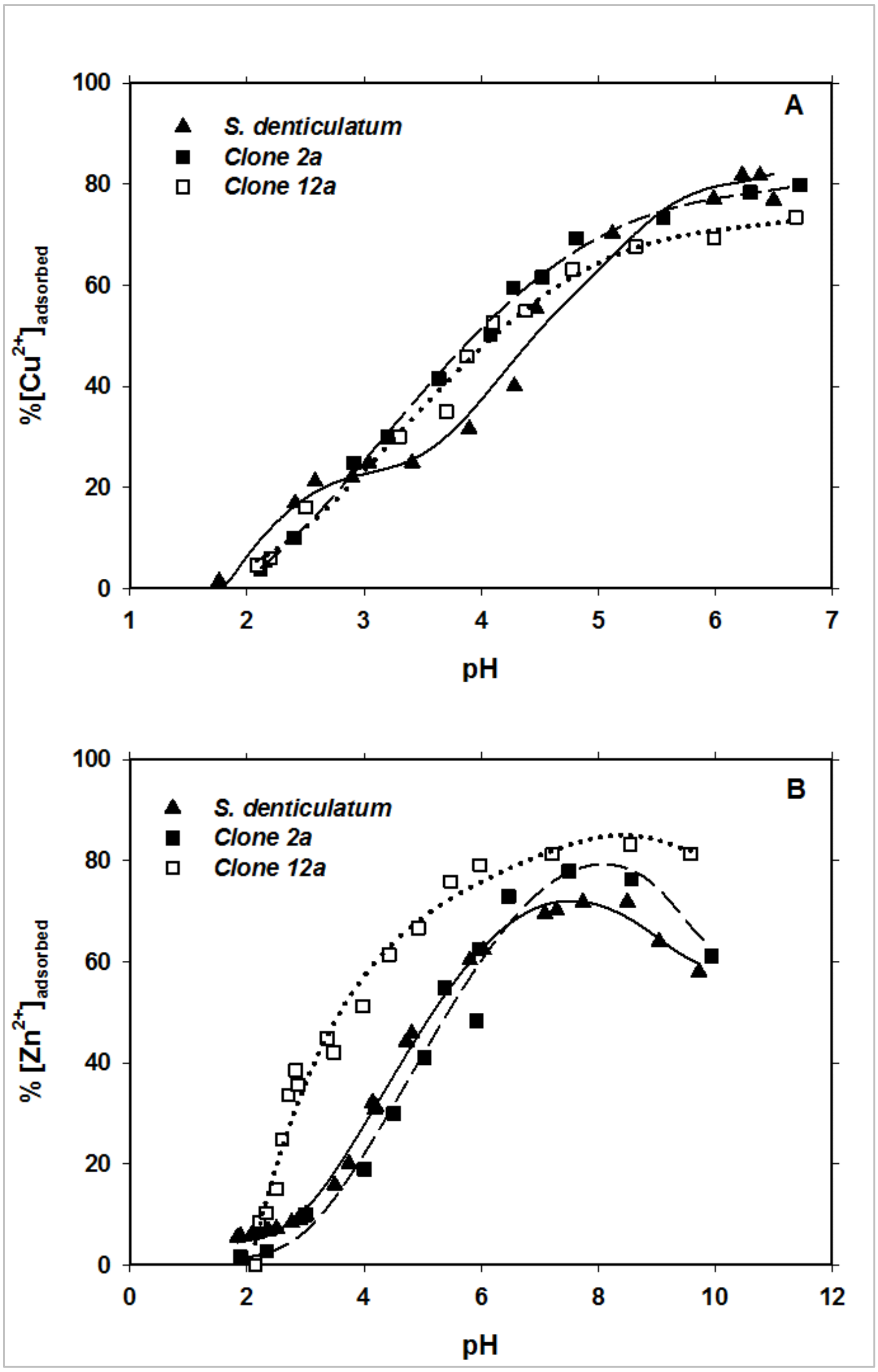


Figure 2. Adsorption of Cu^{2+} (A) and Zn^{2+} (B) onto *S. denticulatum* and *S. palustre* clones as a function of pH, in 0.01 M $NaNO_3$ with constant biomass of 1 g_{dry} L^{-1}. The initial metal loading was 52 and 50.5 µmol L^{-1} for Cu^{2+} and Zn^{2+}, respectively. The lines represent the LPM fit to the data.

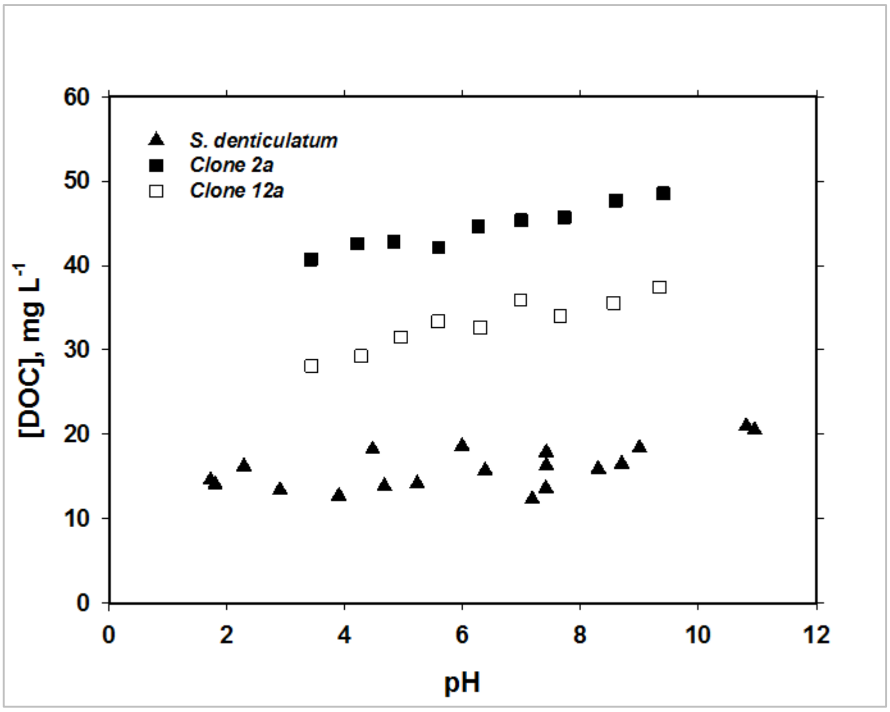


Figure 3. Dissolved Organic Carbon (DOC) excreted by *S. denticulatum* from the field and *S. palustre* clones as a function of pH in 0.01 M $NaNO_3$ with constant biomass of 1 g_{dry} L^{-1}.

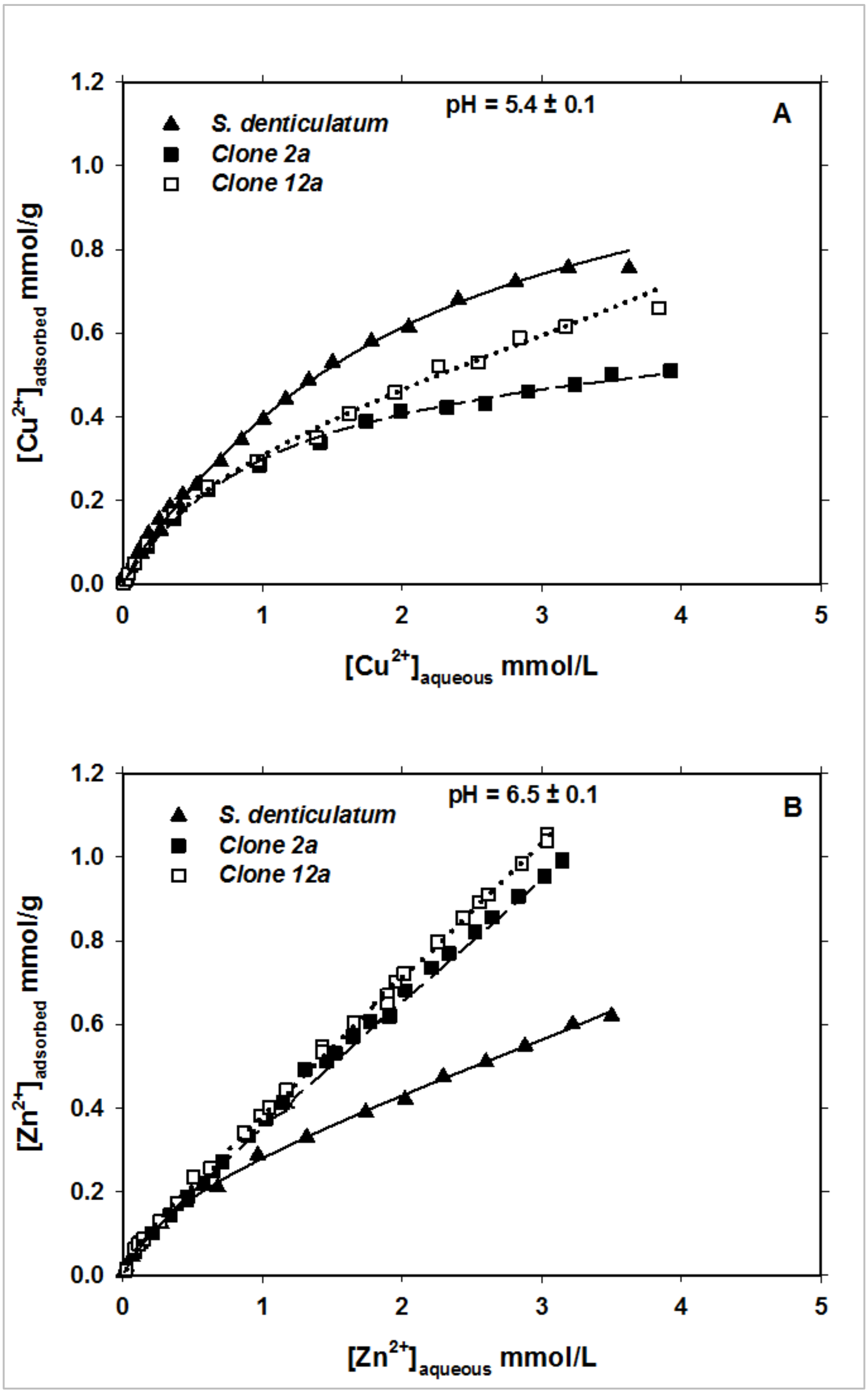


Figure 4. Adsorption of Cu^{2+} (A) and Zn^{2+} (B) onto *S. denticulatum* from the field and *S. palustre* clones as a function of metal concentration in 0.01 M $NaNO_3$ with constant biomass (1 g_{dry} L^{-1}). The pH was fixed at ~5.5 and ~6.5 for Cu^{2+} and Zn^{2+}, respectively. The aqueous metal concentration ranged from 4 to 3920 and from 4 to 3500 µmol L^{-1} for Cu^{2+} and Zn^{2+}, respectively. The lines represent the LPM fit to the data.

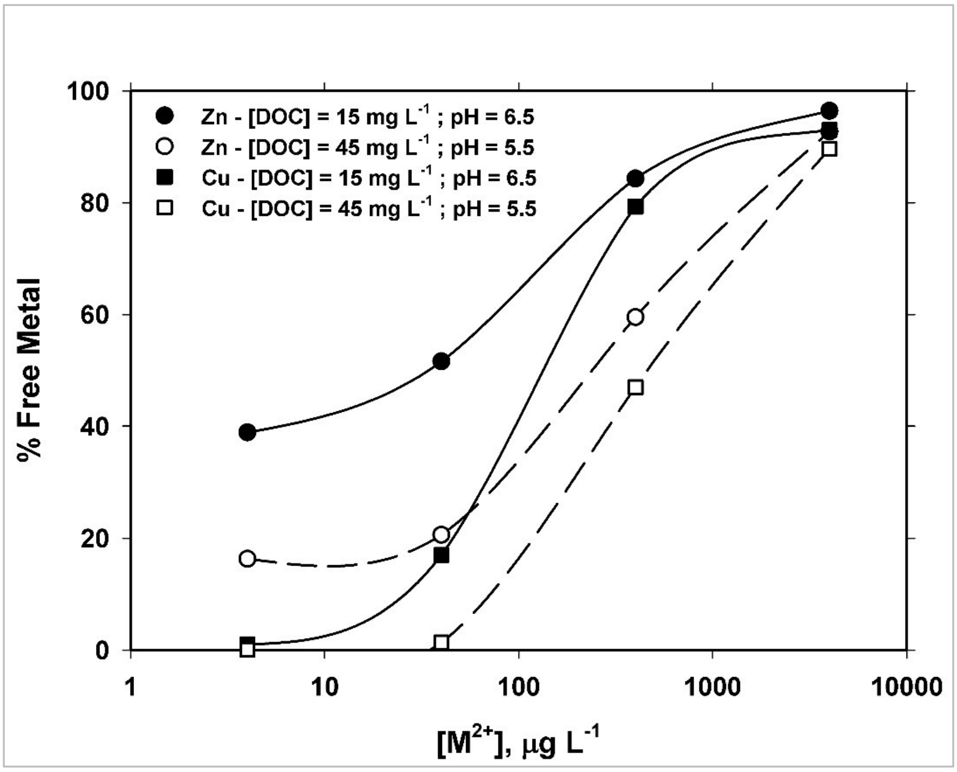
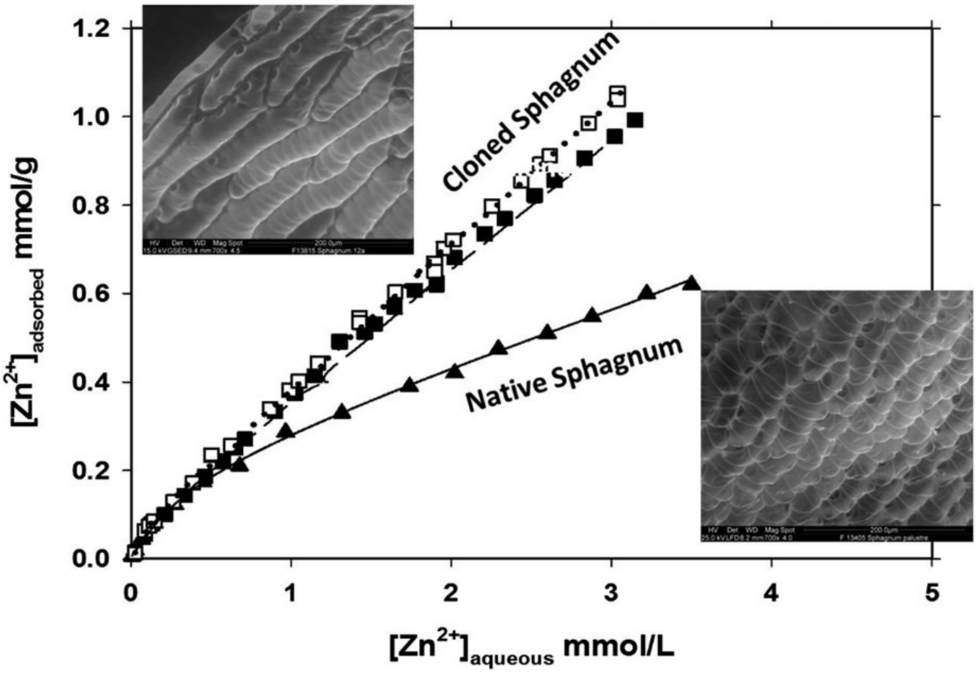


Figure 5. Results of the thermodynamic modeling of Cu^{2+} (squares) and Zn^{2+} (circles) complexation with DOM leached from peat mosses (NICA-Donnan FA complexes in 0.01 M $NaNO_3$), at pH 5.5 and 6.5 for Cu and Zn, respectively. Closed symbols were computed for [DOM] = 15 mg L^{-1} and open symbols are for [DOM] = 45 mg L^{-1}.

Graphical abstract



GENERAL CONCLUSIONS

In order to standardize the moss-bags method for the active biomonitoring of air quality, all the factors influencing the accumulation of pollutants by mosses exposed in bags were for the first time examined separately, taking into account the differences in climate conditions and land uses of the exposure areas. It is proposed to the scientific community a standard protocol for active biomonitoring, by using a new-generation moss-bag, a reusable, not home-made and with specific and fixed characteristics tool. The application of the standard protocol will make biomonitoring surveys directly comparable and it can be more easily recognized and adopted by the member States of the European Union as official monitoring method.

The moss-bags technique, implemented according to the standardized exposure protocol, can be considered a valid approach for a qualitative assessment of air pollution in view of regulatory purposes. It is sensitive for detection of airborne inorganic and organic compounds, also in areas like the Italian Campania Region characterized by a no clear distinction between different land uses and pollution inputs. The moss bag method is very useful to trace all the possible pollutant sources and to detect, even at very small area, a wider set of pollutants than that analysed by the monitoring stations.

For the first time it was shown that it is possible to combine two different methods for the air quality assessment: the emission sources inventory and the active biomonitoring by moss-bags. These combined approaches can be useful for: a) the individuation of all the possible sources of pollutants; b) the detection of pollutants not monitored by automatic devices; c) the control of the pollutant levels and the compliance of the pollution limit values in areas of alleged pollution; d) the realization of mathematical models for monitoring air quality and the planning of monitoring surveys.

The *Sphagnum palustre* clone is an excellent material for the active monitoring of air pollutants. Its *in vitro*-production solves the problem of the severe environmental impact due to the uncontrolled harvesting of field mosses. Unlike the naturally grown moss, the clone is a more homogeneous material, with a very low element content such as to allow the detection of those pollutants present in the environment at very low concentrations, even for short exposure periods. Exposure field test in bags, the comparisons with literature data and the experiments performed for testing the metal adsorption capability showed that the clone *S. palustre* possesses a high ability to adsorb pollutants, with physical-chemical properties and morphological characteristics more suitable to detect airborne pollutants than naturally grown mosses.

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THANKS …

To the truth, to the friendship, to the hope.

To the gentle hands and the kind smiles.

To the sweet words and the happy dances.

To the silence of the innocent nights…

To all the people who believed in that.