UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXVIII CICLO DEL DOTTORATO DI RICERCA IN
BIOLOGIA AMBIENTALE

Methodological aspects about the monitoring of airborne persistent pollutants through the "Moss Bags" approach

Settore scientifico-disciplinare:
BIO/03 - BOTANICA AMBIENTALE E APPLICATA

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ANNO ACCADEMICO 2014 / 2015

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…a Margherita, Sabina e al piccolo che sta per nascere…

FOREWORD

The present work was developed during my collaboration to the European “EU FP7 Mossclone” and “LIFE-ECOREMED” projects, both focused on the methodological aspects of the “moss bags” technique. Although at the end of these work experiences we could demonstrate the high performance of the *Sphagnum palustre* L. clone, a large part of the field experiments were carried out by means of other moss species, due to practical needs (at that time, the cloning of the *S. palustre* was not yet optimised). Only the characterization reported in Chapter 1 was performed using the *S. palustre* clone. For the standardization assays of the moss bags technique (Chapter 2) the moss *Pseudoscleropodium purum* (Hedw.) M.Fleisch was employed. The results described in Chapters 3 and 4 were obtained using the moss *Hypnum cupressiforme* Hedw.

ABSTRACT

Mosses, due to their ability to uptake pollutants, are considered excellent biomonitors. One of the most diffuse method to use them for bio-monitoring purposes is the so called moss bags technique. This practise, consisting in the use of small envelope of plastic net filled with mosses, allows the monitoring of areas lacking the target species. Although this methodology was largely used since the 70’s, it yet suffers of some limitations: 1) a certain environmental impact in the collection sites; 2) the sampling of native mosses is problematic for untrained people; 3) among moss materials of different origins there is an intrinsic variability in elemental and chemical composition; 4) the bag are hand-made prepared; 5) the lack of standardization of the exposure protocol, making derived data sets not directly comparable; 6) the lack of knowledge about the relation existing between the concentrations of pollutants found in the “bioaccumulator” and those estimated by models or using emission inventories; 7) scarcity of information about the ability of the moss bag technique to discriminate the pollution between areas relatively close to each other and characterized by different land uses; 8) so far, it is impossible to estimate the atmospheric content of pollutants by knowing the element concentrations in the exposed mosses.

In lights of the previous issues, four studies have been carried out to provide solutions at the first seven points with the following aims:

a) to define the morphological, molecular and chemical signature of the *S. palustre* clone developed in the framework of the EU-FP7 Mossclone project. To tag the clone, a molecular characterization was performed by a set of DNA molecular markers (RAPD, ISJ, PCR-RFLP, sequencing and microsatellites). The relation between the elemental composition of the clone with the commonly applied pre-exposures treatments (moss oven devitalization and EDTA washing), was estimated by ICP-MS analysis of 54 major and trace elements, and was compared with that of conspecific field samples. In a field test, the metal accumulation ability of bags filled with the clone was compared with respect to that of those filled with *Pseudoscleropodium purum* **(Chapter 1)**;

b) to standardize the moss bags exposure protocol in terms of bag characteristics (shape of the bags, mesh size, weight of moss/surface of bag ratio), duration and height of exposure. The field experiments took place in Austria, Italy and Spain, selecting in each country seven exposure sites affected by different degrees and types of contamination. The different solutions tested (shape, mesh size…) were compared at parity of all the other characteristics and exposure conditions. In this work, the versatility of a new device, the Mossphere (see the Chapter 2 for clarifications), was tested for the first time. For the selection of the best shape, two couplets of moss bags were compared: rounded bag vs. Mossphere and flat bag vs. Mossphere. Three mesh sizes were tested: 1 mm, 2 mm, and 4 mm. Finally, to investigate the third characteristic, three weight/surface ratios of 15, 30 and 45 mg/cm^2 were tested. Both the mesh and weight effects were performed employing the Mossphere. For the selection of the appropriate exposure duration, Mosspheres with a dry mass/surface ratio of 30 mg/cm^2 and 2 mm mesh were exposed in triplicate at 4 m above ground, for 3, 6 and 12 weeks. For the selection of the proper exposure height, 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 **(Chapter 2)**;

c) to compare data provided by the moss bags technique with the inventory of the emissions, one of the methodologies already consolidated and adopted by the EU legislation. The two approaches were simultaneously applied in five Municipalities of the Campania region (Southern Italy), an area where the emission sources are constantly monitored being a National Interest Priority Site **(Chapter** 3**)**;

d) to test the capability of the moss bags in the field to discriminate between the degree of pollution of areas relatively close to each other and characterized by different land uses. Through a specific exposure design, we investigated the capability of moss bags (following an exposure protocol derived from to the results of the above mentioned researches) to distinguish pollution inputs in sites with different land use (agricultural vs. urban/residential) and proximity to roads, in a fragmented landscape of Campania, where areas with different land use are strictly mixed together **(Chapter 4)**.

The pre-treatment tests on the *S. palustre* clone show that the devitalization is useful to preserve the biomaterial and ensures its easy handling. The EDTA washing, although essential for native materials, is unnecessary for the clone since it shows a significantly lower content of elements, also when compared to that of its native counterpart. Furthermore, in the field test, the *S. palustre* clone showed a significantly higher metal uptake compared to *P. purum* (a species largely employed in bio-monitoring assays) (Chapter 1).

The results of the standardization (Chapter 2) evidenced that the shape of the bags and the mesh size did not affected the accumulation capacity of the contained moss, whereas our tests confirmed that the lowest the density of the moss inside the bags, the highest the element uptake. Moreover, three weeks of exposure were not enough to have a consistent accumulation, while only a small difference was observed between the 6- and the 12-week exposure. The height experiment did not evidenced differences among the three proposed solutions. According to the reported outcomes, it

is preferable the use of a Mossphere (being not home-made, reusable and with a standard shape) with a 2 mm mesh and a weight/surface ratio ranging between 5 up to 15 mg cm^{-2} exposed for a period not lower than 6 weeks (i.e. increase temporal resolution) at 4 meters above the ground (this for practical questions).

The last two works (exposed in Chapters 3,4) highlighted the advantages in the joint use of moss bags and emission inventories approaches as a valuable resource to reveal contaminants that are not routinely monitored and also the capability of the moss bags to discriminate pollution inputs at low scale in sites characterized by a fragmented landscape in terms of land use. Particularly, mosses exposed in agricultural sites showed higher pollutant loads than those exposed in urban sites, so as for the mosses hanged at front road sites compared to the matching green ones.

INTRODUCTION

In 2008, indoor air pollution and urban air quality were indicated as two of the major global threats among pollution problems (World’s Worst Pollution Problems report, 2008). More recently, the WHO reported that air pollution in 2012 caused the deaths of about 7 million people worldwide (WHO, 2014). This confirms air pollution as one of the most important environmental health risk in the world and indicates its reduction as an urgent task to save millions of lives.

According to the European Council Directive 96/62/EC on ambient air quality assessment and management and the more recent Directive 2008/50/EC, the member states are obliged to “*set objectives for ambient air quality in order to avoid, prevent or reduce harmful effects on human health and the environment . . . and improve air quality where it is unsatisfactory*". It is very complicated to reach these targets since, before taking actions to improve air quality, an effort should be done to understand the mechanisms of pollutant dispersion and hence realize the best solutions for their monitoring. It is arduous to obtain a completely detailed information on the concentrations and emission forms of pollutants, since their release in the atmosphere is very variable in space and time. It is well known, for instance, that two close sites could be characterized by different air quality and that different monitoring surveys made in the same site but in diverse periods could provide divergent information. Moreover, the automatic devices used nowadays for air monitoring are accurate but too limited in number, due to their excessive costs, to describe the spatial-temporal trends of pollutants. Also, we lack information on the air quality from many places like private homes and work places. To overcome these limitations, it is common to do estimates or use models, based on the few existing measure points; calculating, indirectly, air quality for areas not monitored by any devices. Due to their nature, such procedures are characterized by an intrinsic uncertainty. Besides, the monitoring stations are calibrated to detect just a limited number of pollutants (mainly CO, SO_x, N_xO_y, PAH and $PM_{2.5/10}$), while, the measurement of other air contaminants included in the European Directives (e.g. heavy metals and PAHs) is yet associated to important technical and economic difficulties. Therefore, there is an urgent need to develop and employ new low-cost and robust tools for monitoring air quality.

Biomonitoring is an adequate alternative technique to acquire data about pollution. Biological monitoring, or biomonitoring, is based on the analysis of particular organisms called ‘bio-indicators’, or communities of these organisms, to assess changes in the environment compared to natural conditions. Two different biomonitoring techniques are known: i) bioaccumulation techniques, which measure elemental or chemical concentrations in organisms able to adsorb and accumulate such substances; ii) bioindication techniques, which estimate the effects of pollution on sensitive organisms, which can provide important information about the ecosystem functionality.

This Ph.D. dissertation is focused on the bioaccumulation techniques. A “bioaccumulator” refers to an organism absorbing a toxic substance at a rate faster than that at which the substance is lost. The bioaccumulation technique is either “passive”, i.e. based on the collection of autochthonous species; or “active”, i.e. based on transplants of organisms in areas where they do not spontaneously occur or where they are too rare to be collected in sufficient quantity. Both these techniques offer the following advantages: a) to obtain information on the atmospheric deposition of pollutants, at low costs, with high density of sampling points, and at different spatial scales; b) to identify geographic patterns induced by pollutant transport and deposition, and also evaluate the reliability of dispersion/circulation models; c) to check the effect of the actions applied to minimize pollutant emissions; d) to evaluate temporal trends; e) to identify hot-spots of pollution for planning the location of more accurate instrumental recordings and the development of epidemiological research. The main limits of biomonitoring, especially when working with naturally occurring species (“passive” biomonitoring), are associated with the presence/absence of the target organisms in the investigated areas - mainly due to ecological or pollution factors - which shape the experimental design. Another problem is the lack of knowledge about the correlation existing between the concentrations of pollutants found in the “bioaccumulator” and their concentrations in the atmosphere. This point is still an open issue and further studies are needed in order to reach any steady conclusion. By contrast, to overcome the problem associated with the presence/absence of the target organisms, and to obtain a more rational sample distribution not ruled by this variable, the use of transplants has had great success and increasing employment in the last decades. This approach offers several advantages: i) the exposure of the material according to a rational scheme, ii) the knowledge of pre-exposure values which enables the calculation of enrichment rates, iii) the repetition of the monitoring campaigns, and iv) the possibility to monitor sites lacking naturally occurring species, as are the majority of the most polluted areas (i.e. urban and industrial sites).

Among the available bioaccumulators, bryophytes and in particular mosses are particularly adequate for air quality assessment thanks to their innate characteristics. The nutrient supply of mosses is strictly linked to their poikilohydric method of water regulation (state of hydration controlled by the environment) (Bowen, 1933). They receive water predominantly from rainfall, dew and atmospheric humidity and they can get nutrients from both these channels or directly from dust. Moreover, they are able to resist alive for long periods of dryness, during which their metabolism drastically decreases to restart as soon as water is again available. Mosses can absorb mineral nutrients over their entire surface (Brown, 1982) thanks to a large surface/mass ratio. Besides, mosses have one-cell-layer leaflets, facilitating the exposition of every single cell directly to the nutrient supply. The mosses can grow in situations that could be limiting or prohibitive for other plants, since they are able to take up nutrients even from weak solutions (Babb & Whitfield, 1977). The sequestration of nutrients in mosses occurs through exchange sites (Clymo, 1964; Craigie & Maass, 1966; Wells & Brown, 1990; Bates, 1997), characteristic that varies among species (Büscher et al., 1983). The cation exchange capacity (CEC) appears to be the first step in uptake of nutrient cations (Koedam & Büscher, 1983) and it is allowed by the high concentrations of non-esterified pectates, mostly polyuronic acids, within the cell walls (Clymo, 1963; Craigie & Maass, 1966). Indeed, the concentration of glucuronic acid in bryophytes primary cell walls is higher than those found in any other land plant, as demonstrated by Popper and Fry (2003). The carboxyl group (COOH) of these acids protrudes on the outer surface of the wall, and can freely exchanges its H^+ with other cations in its surroundings. This is particularly evident for *Sphagnum* species for which the nutrient uptake in poor nutrient habitats is further supported by their great ability to exchange Ca^{++} and Mg^{++} ions with H^+ ions (Temple et al., 1981). More recently, González and Pokrovsky (2014) found that *Sphagnum sp.* has the highest proton and metal adsorption capacity in comparison to *Pseudoscleropodium purum* (Hedw.), *Brachytecium rutabulum* (Hedw.), and *Hypnum sp.* Also, the binding of elements or chemical compound by the mosses is not sufficiently selective (Brown & Bates, 1990), hence, the cation exchange process can also result in accumulation of high levels of ionic pollutants, like metals (Brown, 1984).

These characteristics place the mosses among the best options for biomonitoring purposes (Bargagli, 1998). One clear example of their usefulness is offered by the Biomonitoring Network, 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), focused on bio-monitoring of heavy metals and POPs in mosses collected from more than 30 European countries. This network has the aim to assess the sufficiency and effectiveness of the air pollution abatement policies of the LRTAP Convention and its Protocols, and is concentrated on long-range transboundary air pollution (for an overview, see Harmens et al., 2015). This is an example of “passive” bio-monitoring based on the harvesting of naturally growing mosses. However, as highlighted above, it suffers for the absence of target species in urban areas and industrial areas, and even indoor reference environments, making difficult the choosing of a rational sampling scheme. This limit strongly encourages the use of moss transplants.

The most common transplant-based technique is the moss-bags approach described by Goodman and Roberts (1971), the first proposing the transplant of mosses collected from pristine areas by means of small envelops, whose elemental composition after the exposure period is compared to those of unexposed samples (a review in Ares et al., 2012). Goodman and Roberts did not suggest a detailed protocol for the application of the technique, although a well-established procedure is very important in order to compare data obtained in different surveys.

Three main aspects should be taken into account to standardize the “moss-bags” technique. Firstly the choice of the best moss species on the basis of its accumulation capability; indeed, the criterion adopted so far has been a preferential harvesting of those species particularly abundant from unpolluted areas in the surroundings of the survey area; this involves the collection of different moss species (e.g. *Pseudoscleropodium purum* in northern Spain, *Hypnum cupressiforme* in southern Italy and so on). Moreover, it is not always possible to have enough material since its availability depends on natural and anthropogenic causes; even when moss material is available, a repeated harvesting of naturally growing mosses has an important ecological impact. Also, the collection of some species is interdicted by European Directives (e.g. *Sphagnum spp.*). The ability of the operators to identify the target species in the field is another important factor that can be problematic for untrained people. The second problem is the natural variability of moss elemental composition (including pollutants). Often moss species collected in different sites or in the same site but in different seasons have different morphology and elemental composition. Thus, a crucial aspect is the definition of the pre-exposure conditions (e.g. bio-material with a similar morphology, well-characterized initial contents, comparable physiological status; see Godinho et al., 2008). Luckily, in recent years, many advances have been done to standardize these aspects and many studies reported for instance the usefulness of a careful water-washing of moss selected for bag preparation to reduce the variability of bio-monitoring results (e.g. Tretiach et al., 2007, Adamo et al., 2008). In addition, Giordano et al. (2009) found that the oven drying devitalization of moss material eliminates metabolic contribution to element uptake, allowing the elimination of the variability occurring with the exposures in different environments (i.e. different vitality and growth rate, as confirmed by Fernandez et al., 2010). Since the uptake appears to be mainly passive and based on surface interception of pollutants (Tretiach et al., 2011), it is worth to highlight that the oven treatment do not modify the moss tissues and hence its accumulation capability. Once selected a given species, the third step is the standardization of the exposure protocol, in particular the choice of the appropriate bag characteristics (shape of the bag, mesh size, ratio between weight of moss and bag external surface), height and duration of exposure. Although recently Pittao (2007) and Ares et al. (2014) provided important data on some methodological variables to establish a standardized protocol, few works exist in the literature focused on the standardization of the moss bag technique. Nevertheless, the published results should be seen as a good starting point to solve the numerous, still open problems. Some issues are related to the correct choice of the bag characteristics (e.g. the bag preparation is still a home-made affair, the correct mesh size must be defined, etc.), and the exposure modality (height above ground of exposed bags, duration of the exposures, etc.). Firstly, to improve the strength of the results related to the standardization of the technique, it is needed to perform a biomonitoring test in areas characterized by various climatic conditions and also by different degree of pollution in order to ensure the flexibility of the methodology.

The FP7 Mossclone (ENV.2011.3.1.9-1) is a project focused on the standardization of the moss bags technique, having among its aims the selection of a highly performant moss and the establishing of a clone production of this species under highly controlled conditions. Working at this project González and Pokrovsky (2014) quantified the adsorption of heavy metals on 4 moss species frequently employed in the moss bag technique and found that the *Shagnum sp.* was the species with the highest uptake performance. Since harvesting *Shagnum sp.* in the field is prohibited (according to the European Directive 92/43/EEC), Beike et al. (2015) proposed a protocol for cloning *Shagnum palustre* L. in bioreactors under controlled growing conditions (e.g. pH, irradiation, temperature, culture medium). The possibility to employ a clone in the moss bag technique is deemed to supplies a material ready to use with a very constant and low pre-exposure concentrations since the elemental content in mosses is not affected by the environment. Indeed, the chemical composition of the cloned material is supposed to reflect only the chemical nutrients provided during its production. In the framework of the “Mossclone” project were performed the experiments showed in **Chapters 1**, **2** of this thesis. The first work (**Chapter 1**) concerns the chemical characterization and the molecular signature to tag the *S. palustre* clone, developed by Beike et al. (2015); in addition, a field test was performed to compare the metal accumulation ability of bags filled with the clone to those filled with *Pseudoscleropodium purum* (Hedw.) M.Fleisch. The relation between the elemental composition of the clone with the commonly applied pre-exposures treatments (moss oven devitalization and EDTA washing) was estimated by ICP-MS analysis of 54 major and trace elements, and was compared with that of field grown *Sphagnum palustre*. Moreover, the molecular characterization of the clone was performed by a set of DNA molecular markers (RAPD, ISJ, PCR-RFLP, sequencing and microsatellites).

After testing the efficiency of the cloned material for biomonitoring purposes, in **Chapter 2** are showed the results that could help to answer to the question: “*what is the best exposure protocol for a standardized methodology?*” For the experiments, three country characterized by different climatic conditions (Austria, Italy and Spain) were selected. Moreover, in each country mosses were exposed in seven exposure sites affected by different degrees and types of environmental contamination. The variables taken into account were the bag characteristics (shape of the bags, mesh size, weight/surface ratio), the height above the ground of exposed bags, and the duration of exposure. The diverse solutions tested were compared at parity of all the other characteristics and exposure conditions. For the first time, a new (and not “hand-made”) typology of moss bags has been tested: the “Mossphere”. For the selection of the best shape, two couplets of moss bags were compared: rounded bag vs. Mossphere and flat bag vs. Mossphere. Thanks to the versatility of the Mossphere, three mesh sizes (1 mm, 2 mm and 4 mm) were tested, together with three weight/surface ratios (15, 30 and 45 mg/cm^2). For the selection of the appropriate exposure duration, 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 for 3, 6, and 12 weeks. To test the proper exposure height, 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.

Once tested the parameters affecting the moss bags performance, a test was run to verify whether our methodology actually reflect the levels of air pollution likewise other methods already accepted and supported by the current legislation, in order to support the adoption of the standardized moss bag technique by EU or national authorities. In the light of this, **Chapter 3** of this dissertation reports a comparison of the data provided by the moss bags technique exposed in Campania (Southern Italy), with those obtained by an inventory of the emissions, that is one of the methodologies already consolidated and adopted by the EU legislation.

Finally, in **Chapter 4**, the capability of the moss bags to discriminate the degree of pollution between areas relatively close to each other and characterized by different land uses, is considered through a direct field test. Indeed, by adopting a specific exposure design, we investigated the ability 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 these areas are strictly mixed together. It is worth to note that it would be never possible to obtain such detailed data, down to a very small scale (tens of meters), by the sole use of monitoring stations, since their cost would discourage an exposure design similar to that we adopted in this work. The opportunity to perform the experiments described in the last two chapters was possible through the collaboration to the EU project “LIFE11/IT/275 – ECOREMED”.

References:

Adamo P., Bargagli R., Giordano S., Modenesi P., Monaci F., Pittao E., Spagnuolo V., Tretiach M., 2008. Natural and pre-treatments induced variability in the chemical composition and morphology of lichens and mosses selected for active monitoring of airborne elements. Environmental Pollution. 152, 11–9.

Ares A., Aboal J.R., Carballeira A., Giordano S., Adamo P., Fernández J.A., 2012. Moss bag biomonitoring: a methodological review. Science of the Total Environment. 432, 143-158.

Ares A., Fernández J.A., Carballeira A., Aboal J.R., 2014. Towards the methodological optimization of the moss bag technique in terms of contaminants concentrations and replicability value. Atmospheric Environment. 94, 496–507.

Babb T. A., Whitfield D. W. S., 1977. Mineral nutrient cycling and limitation of plant growth in the Truelove Lowland ecosystem. In: Bliss, L. E. (ed.). Truelove Lowland, Devon Island, Canada: A High Arctic Ecosystem. University of Alberta Press, Edmonton, pp. 589-606.

Bates J. W., 1997. Effects of intermittent desiccation on nutrient economy and growth of two ecologically contrasted mosses. Annals of Botany. 79, 299-309.

Bargagli R. Trace elements in terrestrial plants, 1998. Berlin: Springer.

Beike A.K., Spagnuolo V., Lüth V., Steinhart F., Ramos-Gomez J., Krebs M., Adamo P., Rey-Asensio A.I., Fernandez J.A., Giordano S., Decker E.L., Reski R,. 2015. Clonal in vitro propagation of peat mosses (*Sphagnum L.)* as novel green resources for basic and applied research. Plant Cell, Tissue and Organ Culture. 120, 1037-1049.

Bowen E. J., 1933c. The mechanism of water conduction in the Musci considered in relation to habitat. III. Mosses growing in dry environments. Annals of Botany. 47, 889-912.

Brown D. H., 1982. Mineral nutrition. In: Smith, A. J. E. (ed.). Bryophyte Ecology, Chapman & Hall, London, pp. 383-444.

Brown D. H., 1984. Uptake of mineral elements and their use in pollution monitoring. In: Dyer, A. F. and Duckett, J. G. (eds.). The Experimental Biology of Bryophytes, Academic Press, New York, London, pp. 229-256.

Brown D. H., Bates J. W., 1990. Bryophytes and nutrient cycling. J. Botanical Journal of the Linnean Society. 104, 129-147.

Büscher P., Koedam N., Neirinck L., 1983. Cationexchange capacity of mosses in relation to soil preference. In: Cran W. J., Janácek K., Rybová R., and Sigler, K. (eds.). Membrane Transport in Plants. Proc. Symp. held in Prague, Czechoslovakia Aug. 15-21, 1983. John Wiley & Sons, New York, pp. 477-478.

Clymo R. S., 1963. Ion exchange in *Sphagnum* and its relation to bog ecology. Annals of Botany. 27, 309-324.

Clymo R. S., 1964. The origin of acidity in *Sphagnum* bogs. Bryologist. 67, 427-431.

Craigie J. S., Maass W. S. G., 1966. The cation-exchanger in *Sphagnum spp.* Annals of Botany. 30, 153-154.

EU Directive 96/62/EC of 27 September 1996 on ambient air quality assessment and management. Official Journal. (21.11.1996, L296/55)

EU Directive 2008/50/EC of the European Parliament and of the Council of 21 May 2008 on ambient air quality and cleaner air for Europe. Official Journal. (11.06.2008, L152).

Fernández J.A., Ares A., Rey-Asensio A.I., Carballeira A., Aboal J.R., 2010. Effect of growth on active biomonitoring with terrestrial mosses. Journal of Atmospheric Chemistry. 63, 1-11.

Godinho R.M., Wolterbeek H.Th., Verburg T., Freitas M.C., 2008. Bioaccumulation behaviour of transplants of the lichen *Flavoparmelia caperata* in relation to total deposition at a polluted location in Portugal. Environmental Pollution. 151, 318–325.

González A.G., Pokrovsky O.S., 2014. Metal adsorption on mosses: Toward a universal adsorption model. Journal of Colloid and Interface Science. 415, 169-178.

Giordano S., Adamo P., Monaci F., Pittao E., Tretiach M., Bargagli R., 2009. Bags with oven-dried moss for the active monitoring of airborne trace elements in urban areas. Environmental Pollution.157, 2798–805.

Goodman G.T., Roberts T.M., 1971. Plants and soils as indicators of metals in the air. Nature (Lond.), 231–287.

Harmens H., Mills G., Hayes F., Norris D.A., Sharps K., 2015. Twenty eight years of ICP Vegetation: an overview of its activities. Annals of Botany. 5, 31–43

Koedam N. and Büscher P., 1983. Studies on the possible role of cation exchange capacity in the soil preference of mosses. Plant Soil. 70, 77–93.

Pittao E., 2007. Aspetti metodologici del biomonitoraggio di inquinanti persistenti aerodiffusi mediante muschi e licheni. Trieste: Università degli Studi di Trieste, Tesi di Dottorato.

Popper Z. A., Fry S. C., 2003. Primary cell wall composition of bryophytes and charophytes. Annals of Botany. 91, 1-12.

Temple P. J., McLaughlin D. L., Linzon S. N., Wills R., 1981. Moss bags as monitors of atmospheric deposition. Journal of the Air Pollution Control Association. 31, 668-670.

Tretiach M., Adamo P., Bargagli R., Baruffo L., Carletti L., Crisafulli P., Giordano S., Modenesi P., Orlando S., Pittao E., 2007. Lichen and moss bags as monitoring devices in urban areas. Part I: influence of exposure on sample vitality. Environmental Pollution. 146, 380–91.

Tretiach M., Pittao E., Crisafulli P., Adamo, P., 2011. Influence of exposure sites on trace element enrichment in moss-bags and characterization of atmospheric deposition on the biomonitor surface. Science of the Total Environment 409, 822-830.

Wells J. M., Brown D. H. 1990. Ionic control of intracellular and extracellular Cd uptake by the moss *Rhytidiadelphus squarrosus* (Hedw.) Warnst. New Phytologist. 116, 541-553.

Sitography:

[1] http://www.worstpolluted.org/

[2] http://www.who.int/mediacentre/news/releases/2014/air-pollution/en/

[3] 92/43/EEC: http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A31992L0043

Chapter 1

Submitted to Ecological indicators

Submission date 18/01/2016

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.

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).

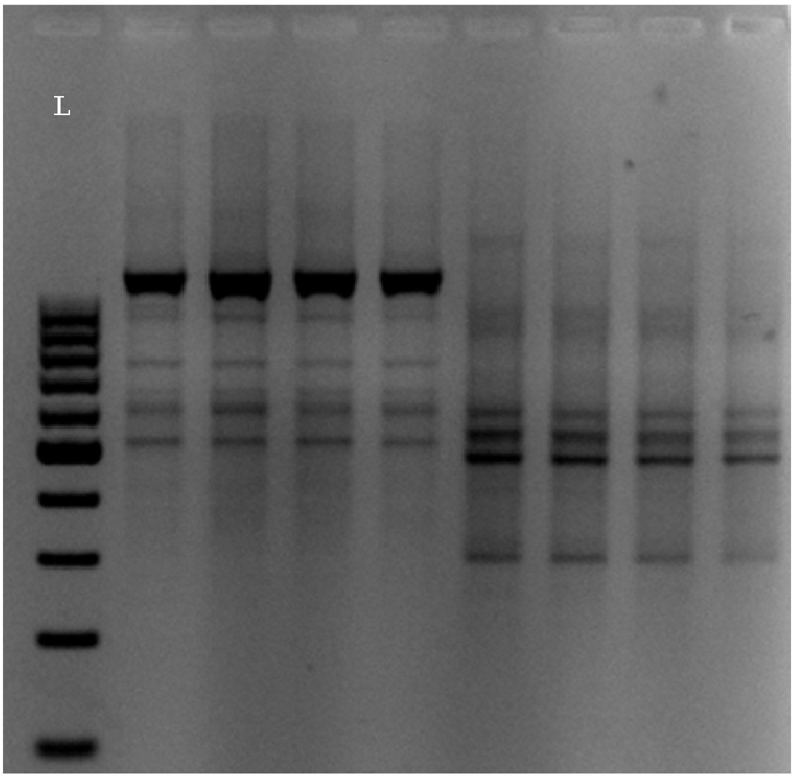

Figure 1. RAPD amplification of the clone 12a; L = ladder 100 bp; lanes 2 to 5: DNA amplifications of 4 different shoots by OPB15 primer; lanes 6 to 9: DNA amplifications of 4 different shoots by OPJ19 primer.

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.

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	-

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.

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	

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.

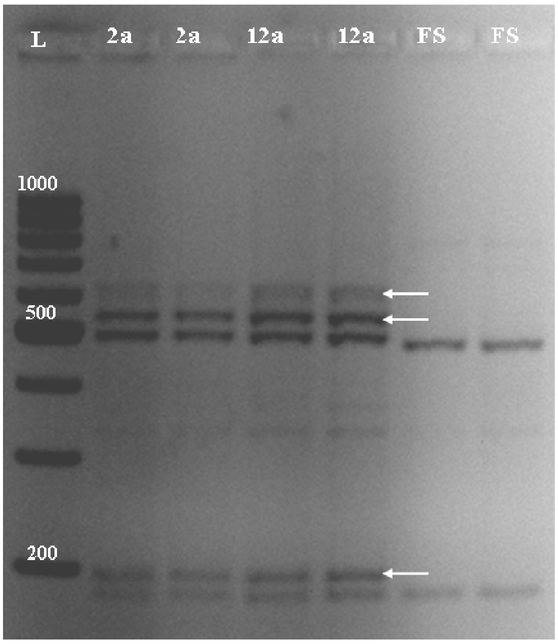

Figure 2. Double digestion by Hind III/HinfI of RAPDf DNA region. L = ladder 100 bp; 2a and 12a are two different lines of *S. palustre* clone; FS = field shoots. Each digestion was performed on a different DNA extractions.

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.

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

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.

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

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.

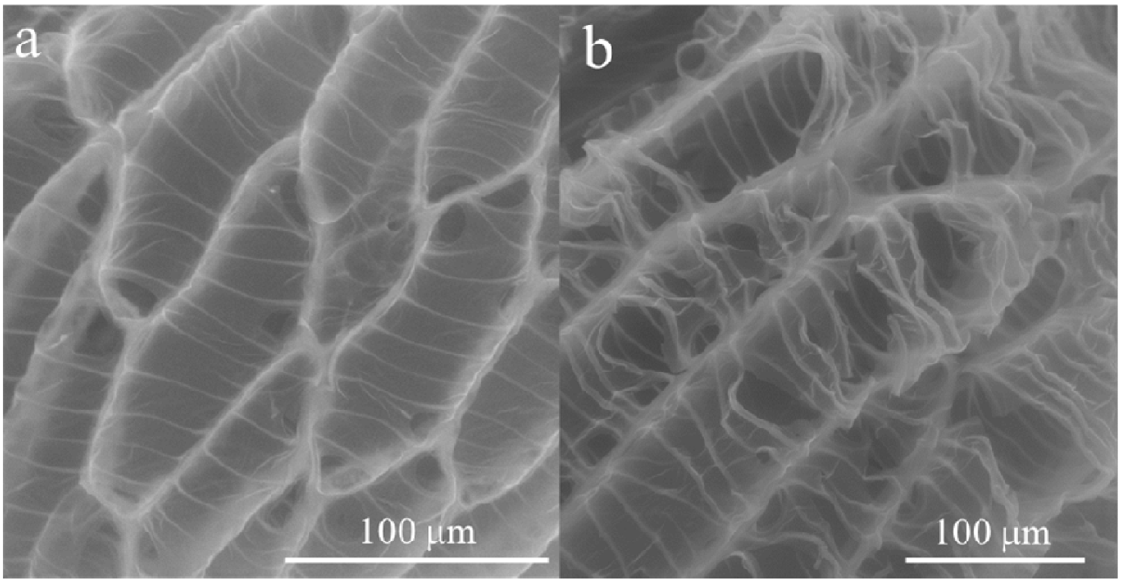

Figure 3. Scanning electron micrographs of *S. palustre* clone before (a) and after EDTA treatment (b).

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.

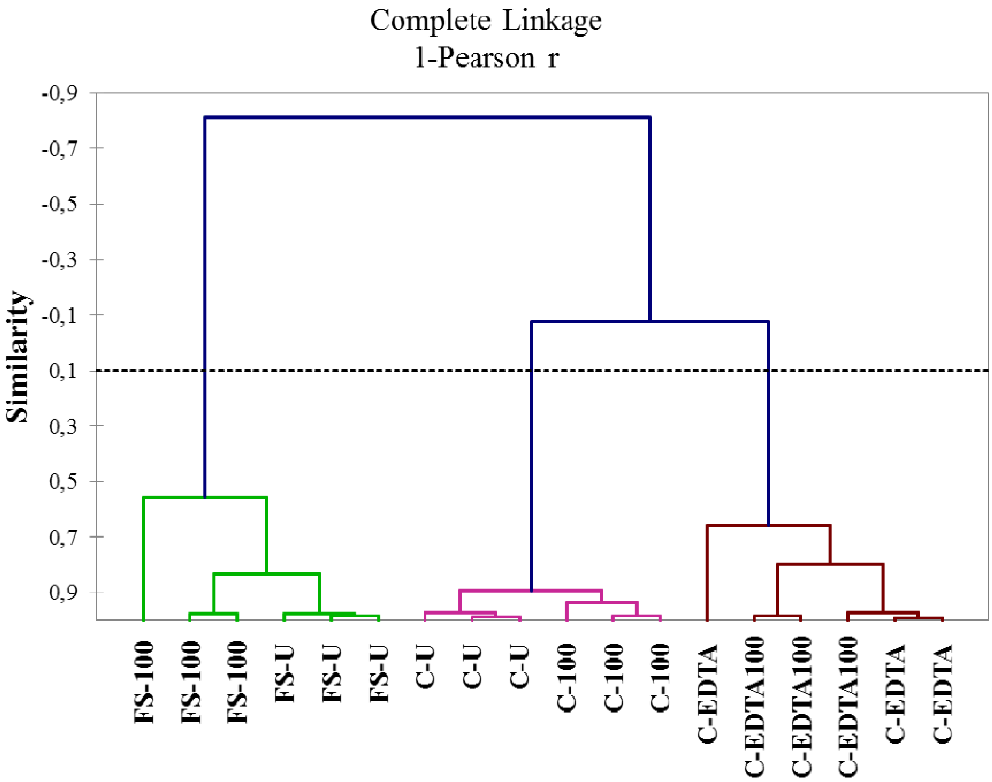

Figure 4. Cluster Analysis of the element concentration data related to FS and C *Sphagnum* samples. For the labels see paragraph 2.2.1.

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.

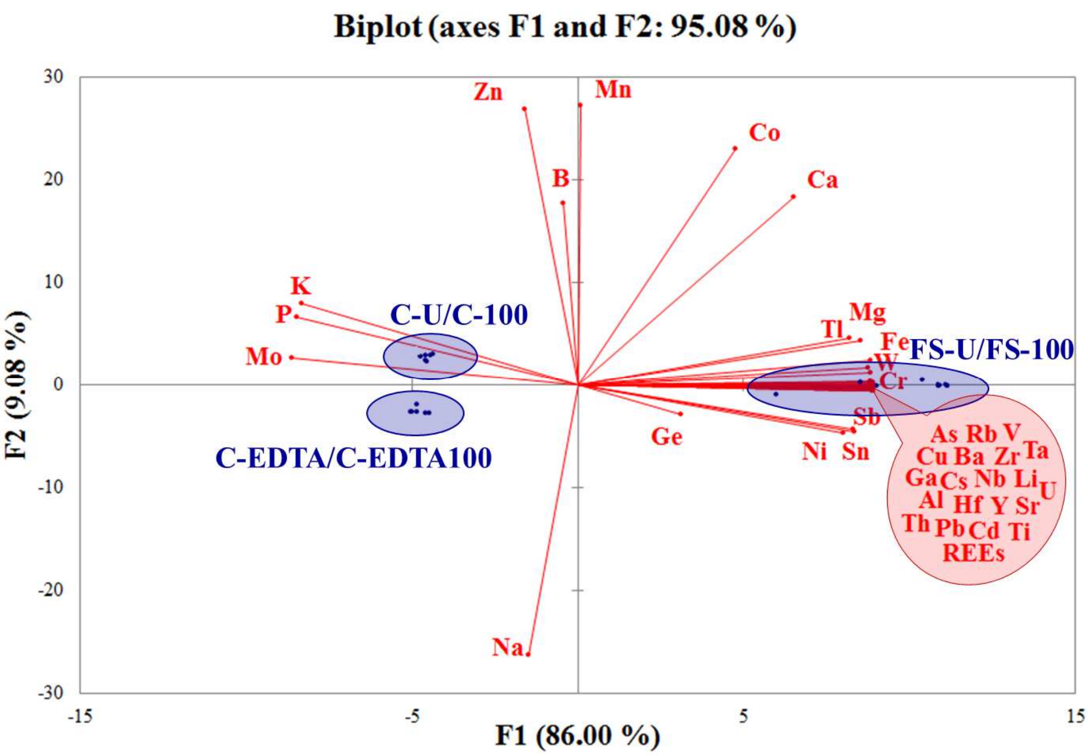

Figure 5. Biplot of Principal Component Analysis for FS and C *S. palustre* data. For the label-codes see paragraph 2.2.1.

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.

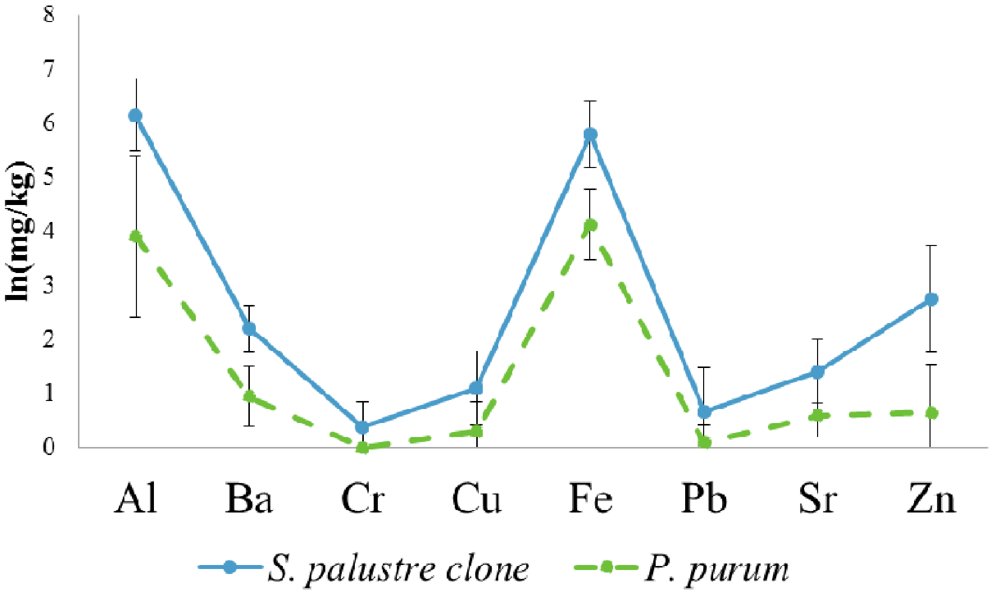

Figure 6. Accumulation values of Al, Ba, Cr, Cu, Fe, Pb, Sr and Zn related to *S. palustre* clone and *P. purum* exposed in bags for 6 weeks at ten Italian and Spanish sites. The values, expressed in ln(mg kg^{-1}) ± ln(SD) as error bars, are the averages, for each element, of the post- minus pre-exposure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

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.

Acknowledgements

This work was funded by FP7-ENV.2011.3.1.9-1 (MOSSCLONE). Partial support from BIO-GEO-CLIM grant RF No 14.B25.31.0001 to O.P. is also acknowledged. We would dedicate this paper to the memory of Stefano Terracciano, who contributed to the molecular characterization of the *S. palustre* clone.

References:

Adamo P., Giordano S., Minganti V., Modenesi P., Monaci F., Pittao E., Tretiach M., Bargagli R., 2007. Lichen and moss bags as monitoring device in urban areas. Part II: Trace element content in living and dead biomonitors and comparison with synthetic materials. Environ. Pollut. 146, 392–399.

Adamo P., Bargagli R., Giordano S., Modenesi P., Monaci F., Pittao E., Spagnuolo V., Tretiach M., 2008a. Natural and pretreatments induced variability in the chemical composition and morphology of lichens and mosses selected for active monitoring of airborne elements. Environ. Pollut. 152, 11–19.

Adamo P., Giordano S., Naimo D., Bargagli R., 2008b. Geochemical properties of airborne particulate matter (PM10) collected by automatic device and biomonitors in a Mediterranean urban environment. Atm. Environ. 42, 346–357.

Aničić M., Tasić M., Frontasyeva M.V., Tomašević M., Rajšić S., Mijić Z., Popović A., 2009. Active moss biomonitoring of trace elements with Sphagnum girgensohnii moss bags in relation to atmospheric bulk deposition in Belgrade, Serbia. Environ. Pollut. 157, 673-679.

Ares A., Aboal J.R., Carballeira A., Giordano S., Adamo P., Fernández J.A, 2012. Moss bag biomonitoring: a methodological review. Sci. Total Environ. 432, 143-158.

Ares A., Fernández J.A., Carballeira A., Aboal J.R., 2014. Towards the methodological optimization of the moss bag technique in terms of contaminants concentrations and replicability value. Atmos. Environ. 94, 496–507.

Archibold O.W., Crisp P.T., 1983. The distribution of airborne metals in the Illawarra region of New South Wales, Australia. Appl. Geogr. 3, 331–344.

Bargagli, 1998. Trace elements in terrestrial plants. An ecophysiological approach to biomonitoring and biorecovery. Springer-Verlag, Berlin, pp. 324.

Bates J.W., 2000. Mineral nutrition, substratum ecology, and pollution. In: Shaw J.A., Goffinet B., (Eds.), Bryophyte Biology. Cambridge: Cambridge University Press; pp. 248-311.

Beike A.K., Spagnuolo V., Lüth V., Steinhart F., Ramos-Gomez J., Krebs M., Adamo P., Rey-Asensio A.I., Fernàndez J.A., Giordano S., Decker E.L., Reski R., 2015. Clonal in vitro propagation of peat mosses (Sphagnum L.) as novel green resources for basic and applied research. Plant Cell Tissue Organ Cult. 120(3), 1037–1049.

Castello M., 2007. A comparison between two moss species used as transplants for airborne trace element biomonitoring in NE Italy. Environ Monit Assess. 133(1-3), 267–276.

Chen Y.E., Cui J.M., Yang J.C., Zhang Z.W., Yuan M., Song C., Yang H., Liu H.M., Wang C.Q., Zhang H.Y., Zeng X.Y, Yuan S., 2015. Biomonitoring heavy metal contaminations by moss visible parameters. J. Hazard. Mater. 296, 201–209.

Couto J.A., Aboal J.R., Fernández J.A., Carballeira A., 2004. A new method for testing the sensitivity of active biomonitoring: an example of its application to a terrestrial moss. Chemosphere. 57(4), 303–8.

Crespo Pardo D., Terracciano S., Giordano S., Spagnuolo V., 2014. Molecular markers based on PCR methods: a guideline for mosses. Cryptogam. Bryol. 35(3), 229-246.

Culicov O.A., Yurukova L., 2006. Comparison of element accumulation of different moss and lichen bags exposed in the city of Sofia (Bulgaria). J. Atmos. Chem. 55, 1-12.

Fernández J.A., Ares A., Rey-Asensio A., Carballeira A., Aboal J.R., 2010. Effect of growth on active biomonitoring with terrestrial mosses. J. Atmos. Chem. 63, 1–11.

Ferreira D., Ciffroy P., Tusseau-Vuillemin M.H., Garnier C., Garnier J.M., 2009. Modelling exchange kinetics of copper at the water-aquatic moss (Fontinalis antipyretica) interface: Influence of water cationic composition (Ca, Mg, Na and pH). Chemosphere. 74(8), 1117-1124.

Giordano S., Adamo P., Sorbo S., Vingiani S., 2005. Atmospheric trace metal pollution in the Naples urban area based on results from moss and lichen bags. Environ. Pollut. 136, 431–442.

Giordano S., Adamo P., Monaci F., Pittao E., Tretiach M., Bargagli R., 2009. Bags with oven-dried moss for the active monitoring of airborne trace elements in urban areas. Environ. Pollut. 157, 2798–805.

González A.G., Pokrovsky O.S., 2014. Metal adsorption on mosses: Toward a universal adsorption model. J. Colloid Interface Sci. 415, 169-178.

González A.G., Pokrovsky O.S., Beike K.A., Reski R., Di Palma A., Adamo P., Giordano S., Fernàndez J.A., 2016. Metal and proton adsorption capacities of natural and cloned Sphagnum mosses. J. Colloid Interface Sci. 461, 326-334.

Harmens H., Mills G., Hayes F., Norris D.A., Sharps K., 2015. Twenty eight years of ICP Vegetation: an overview of its activities. Annali di Botanica 5, 31-43.

Harmens H., Norris D.A., Steinnes E., Kubin E., Piispanen J., Alber R., Aleksiayenak Y., Blum O., Coşkun M. M. Dam M, De Temmerman L., Fernández J.A., Frolova M., Frontasyeva M., González-Miqueo L., Grodzińska K., Jeran Z., Korzekwa S., Krmar M., Kvietkus K., Leblond S., Liiv S., Magnússon S.H., Maňkovská B., Pesch R., Rühling Å., Santamari, J.M., Schröder W., Spiric Z., Suchara I., Thöni L., Urumov V., Yurukova L., Zechmeister H.G., 2010. Mosses as biomonitors of atmospheric heavy metal deposition: spatial and temporal trends in Europe. Environ. Pollut. 158, 3144-3156.

Iodice P., Adamo P., Capozzi F., Di Palma A., Senatore A., Spagnuolo V., Giordano S., 2016. Air pollution monitoring using emission inventories combined with the moss bag approach. Sci. Total Environ. 541, 1410-1419.

Lehmann E.L., 1975. Nonparametrics: Statistical Methods Based on Ranks. San Francisco, Holden Day.

Liu Y., Yan H.F., Cao T., Ge X.J., 2010. Evaluation of 10 plant barcodes in Bryophyta (Mosses). J. Syst. Evol. 48, 36–46.

Lodenius M., Tulisalo E., 1984. Environmental mercury contamination around a chlor-alkali plant. Bull. Environ. Contam. Toxicol. 32, 439–444.

Podani J., 2000. Introduction to the Exploration of Multivariate Biological Data. Backhuys Publishers, Leiden.

Sawicki J., Szczecińska M., 2007. Semi-specific intron-exon splice junction markers in bryophyte studies. Biodiv. Res. Conserv. 5(8), 25–30.

Sawicki J., Plášek V., Szczecińska M., 2009. Molecular evidence does not support the current division of Orthotrichum subgenus Gymnoporus. Plant Syst. Evol. 279(1-4), 125–137.

Sawicki J., Szczecińska M., 2011. A comparison of PCR-based markers for the molecular identification of Sphagnum species of the section Acutifolia. Acta Soc. Bot. Pol. 80(3), 185–192.

Shaw A.J., Cox C.J., Boles S.B., 2003. Polarity of peat moss (Sphagnum) evolution: who says Bryophytes have no roots? Am. J. Bot. 90(12), 1777-1787.

Shaw A.J., Cao T., Wang L-S., Flatberg K.I., Flatberg B., Shaw B., Zhou P., Boles S.B., Terracciano S., 2008. Genetic variation in three Chinese peat moss (Sphagnum) based on microsatellite markers, with primer information and analysis of ascertainment bias. Bryologist. 111(2), 271–281.

Skotniki M.L., Ninham J.A., Selkirk P.M., 1999. Genetic diversity and dispersal of the moss Sarconeurum glaciale on Ross Island, East Antarctica. Mol. Ecol. 8, 753–762.

Spagnuolo V., Giordano S., Pérez-Llamazares A., Ares, A., Carballeira A., Fernández J.A., Aboal J.R., 2013. Distinguishing metal bioconcentration from PM in moss tissue: testing methods of removing particles attached to the moss surface. Sci. Total Environ. 463–464, 727–733.

Stepanova V.M., Pokrovsky O.S., Viers J., Mironycheva-Tokareva N.P., Kosykh N.P., Vishnyakova E.K., 2015. Major and trace elements in peat profiles in Western Siberia: impact of the landscape context, latitude and permafrost coverage. Appl. Geochem. 53, 53–70.

Terracciano S., Giordano S., Bonini I., Miserere L., Spagnuolo V., 2012. Genetic variation and structure in endangered populations of Sphagnum palustre L. in Italy: a molecular approach to evaluate threats and survival ability. Botany. 90, 966–975.

Tretiach M., Adamo P., Bargagli R., Baruffo L., Carletti L., Crisafulli P., Giordano S., Modenesi P., Orlando S., Pittao E., 2007. Lichen and mossbags as monitoring devices in urban areas. Part I: influence of exposure on sample vitality. Environ. Pollut. 146, 380–91.

Tretiach M., Pittao E., Crisafulli P., Adamo P., 2011. Influence of exposure sites on trace element enrichment in moss-bags and characterization of particles deposited on the biomonitor surface. Sci. Total Environ. 409, 822–830.

Vanderpoorten A., Hedenas L., Jacquemart A.L., 2003. Differentiation in DNA fingerprinting and morphology among species of the pleurocarpous moss genus, Rhytidiadelphus (Hylocomiaceae). Taxon. 52(2), 229–236.

Viers J., Oliva P., Sonke J., Nonell A., Freydier R., Gainville R., Dupré B., 2007. Evidence of Zn isotopic fractionation in a soil-plant-system of a pristine tropical watershed (Nsimi, south Cameroon). Chem. Geol. 239, 124–137.

Viers J., Prokushkin A.S., Pokrovsky O.S., Kirdyanov A.V., Beaulieu E., Zouiten C., Oliva P., Dupré B., 2013. Seasonal and spatial variability of elemental concentrations in boreal forest larch foliage of Central Siberia on continuous permafrost. Biogeochemistry. 113, 435–449.

Vingiani S., Adamo P., Giordano S., 2004. Sulphur, nitrogen and carbon content of Sphagnum capillifolium and Pseudevernia furfuracea exposed in bags in the Naples urban area. Environ. Pollut. 129, 145–158.

Vingiani S., De Nicola F., Purvis W.O., Concha-Graña E., Muniategui-Lorenzo S., López-Mahía P., Giordano S., Adamo S., 2015. Active Biomonitoring of heavy metals and PAHs with mosses and lichens: a case study in the cities of Naples and London. Water Air Soil Pollut. 226(8), 240.

Zar J.H., 2010. Biostatistical Analysis. 5th ed. Prentice-Hall, Englewood Cliffs, NJ.

Zechmeister H.G., Hohenwallner D., Riss A., Hanus-Illnar A. 2003. Variations in the moss species Abietinella abietina (Hedw.) Fleisch. according to sampling time, within site variability and increase in biomass. Sci. Total Environ. 301, 55-65.

Sitography

1999/30/EC:
http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A31999L0030.
2002/3/EC:
http://eur-ex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:067:0014:0030:EN:PDF
2004/107/EC:
http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2005:023:0003:0016:EN:PDF
2008/50/EC:
http://eur-lex.europa.eu/legal-content/en/ALL/?uri=CELEX:32008L0050
92/43/EEC:
http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A31992L0043
CCEP -COM/2013/0918:
(http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:52013DC0918

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. cristatum[2]	New Zeland						0.0007					0.0039				2						0.0025	
S. girgensohnii[3]	Russia	254	0.11		17			0.38	0.37	0.26	0.31					297				0.19		114	
S. girgensohnii[4]	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[5]	Russia	254	0.11				0.18			0.25		2.1				297						113	
S. girgensohnii[6]	Finland															132							
S. girgensohnii[7]	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[8]	Russia	347	0.13		20		0.43	0.62	0.28	1.4	0.43					219			37	0.26		372	0.086
S. girgensohnii[8]	Bulgaria	1187	0.52		44		1.1	1	0.3	1.6	0.27					665			63	0.65		302	0.11
S. girgensohnii[9]	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[10]	Bulgaria	600					0.54		0.4			6.3				1200						198	
S. capillifolium[11]	Italy	1108	0.11				0.38		0.43	1.6		5.54				675						552	0.41
S. capillifolium[12]	Italy	1108	0.11				0.38		0.43	1.6		5.54				675						552	0.41
S. capillifolium[13]	Italy						0.9			1.37		4.3				506							
S. angustifolium[14]	Canada	537		4.8	233				0.2	1.1		8.2				383						10.5	0.5
S. auriculatum[15]	Portugal									0.2		7.5				156						0.22	
S. teres[10]	Bulgaria	500					0.48		0.2			5.1				600						246	
S. fuscum[16]	Canada																						
S. fuscum[17]	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[18]	Scotland																						
S. subsecundum[18]	Scotland																						
S. fallax[19]	Poland						0.25																
S. fallax[20]	Poland						0.16																
Sphagnum sp.[21]	Wales						2.9					3.8											
Sphagnum sp.[22]	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. cristatum[2]	New Zeland			0.0001																			0.023
S. girgensohnii[3]	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[4]	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[5]	Russia		2.4	2.2															0.54				20
S. girgensohnii[6]	Finland																						
S. girgensohnii[7]	Bulgaria		1.4			15	0.095	0.14		26	0.025	0.014		0.19	66			0.097	2.1				36
S. girgensohnii[8]	Russia		2.5			29	0.056	34		16	7			45				0.022	1	1			27
S. girgensohnii[8]	Bulgaria		2.1			12	0.085	88		41	21			140				0.057	3	0.52			60
S. girgensohnii[9]	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[10]	Bulgaria		2.5	24																			35
S. capillifolium[11]	Italy		2.4	18.9											11.46				1.55				83
S. capillifolium[12]	Italy		2.4	18.9											11.46				1.55				83
S. capillifolium[13]	Italy		1.23	29.9															2.15				98
S. angustifolium[14]	Canada		0.8	0.1											22.3				1.3	1			46
S. auriculatum[15]	Portugal		3.1	30																			71
S. teres[10]	Bulgaria		2.3	19																			62
S. fuscum[16]	Canada			5.7																			20
S. fuscum[17]	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[18]	Scotland			19.6																			
S. subsecundum[18]	Scotland			20.1																			
S. fallax[19]	Poland			4																			27
S. fallax[20]	Poland			2.5																			
Sphagnum sp.[21]	Wales			4.9																			
Sphagnum sp.[22]	England			6.2																			

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

[1]Szczepzniak et al., 2007; [2]Archibold and Crisp, 1983; [3]Aničić et al., 2008; [4]Aničić et al., 2009a; [5]Aničić et al., 2009b; [6]Kupiainen and Tervahattu, 2004; [7]Culicov and Yurukova, 2006; [8]Culicov et al., 2005; [9]Vuković et al., 2015; [10]Yurukova and Ganeva, 1997; [11]Adamo et al., 2003; [12]Giordano et al., 2005; [13]Vingiani et al., 2015; [14]Archibold, 1985; [15]Vasconcelos and Tavares, 1998; [16]Goodarzi et al., 2002; [17]Calabrese et al., 2015; [18]Ratcliffe, 1975;

References of Table S1

Adamo P., Giordano S., Vingiani S., Castaldo Cobianchi R., Violante P., 2003. Trace element accumulation by moss and lichen exposed in bags in the city of Naples (Italy). Environ. Pollut. 122, 91-103.

Al-Radady A.S., Davies B.E., French M.J., 1994. Distribution of lead inside the home: cases studies in the north of England. Sci. Total Environ. 145, 143-56.

Aničić M., Tasić M., Frontasyeva M.V., Tomašević M., Rajšić S., Strelkova L.P., Popović A., Steinnes E., 2008. Active biomonitoring with wet and dry moss: A case study in an urban area. Environ. Chem. Lett. 7, 55-60.

Aničić M., Tomašević M., Tasić M., Rajšić S., Popović A., Frontasyeva M.V., Lierhagen S., Steinnes E., 2009a. Monitoring of trace element atmospheric deposition using dry and wet moss bags: Accumulation capacity versus exposure time. J. Hazard. Mater. 171, 182-188.

Aničić M., Tasić M., Frontasyeva M.V., Tomašević M., Rajšić S., Mijić Z., Popović A., 2009b. Active moss biomonitoring of trace elements with Sphagnum girgensohnii moss bags in relation to atmospheric bulk deposition in Belgrade, Serbia. Environ. Pollut. 157, 673-679.

Archibold O.W., 1985. The metal content of wind-blown dust from uranium tailings in northern Saskatchewan. Wat. Air Soil Pollut. 24, 63-76.

Archibold O.W., Crisp P.T., 1983. The distribution of airborne metals in the Illawarra region of New South Wales, Australia. Appl. Geogr. 3, 331-344.

Calabrese S., D'Alessandro W., Bellomo S., Brusca L., Martin R.S., Saiano F., Parello F., 2015. Characterization of the Etna volcanic emissions through an active biomonitoring technique (moss-bags): part 1 - major and trace element composition. Chemosphere. 119, 1447-55.

Culicov O.A., Mocanu R., Frontasyeva M.V., Yurukova L., Steinnes E,. 2005. Active moss biomonitoring applied to an industrial site in Romania: relative accumulation of 36 elements in moss-bags. Environ. Monit. Assess. 108, 229-240.

Culicov O.A., Yurukova L., 2006. Comparison of element accumulation of different moss and lichen bags exposed in the city of Sofia (Bulgaria). J. Atmos. Chem. 55, 1-12.

Dmuchowski W., Bytnerowicz A., 2009. Long-term (1992–2004) record of lead. cadmium. and zinc air contamination in Warsaw, Poland: determination by chemical analysis of moss bags and leaves of Crimean linden. Environ. Pollut. 157, 3413-3421.

Dmuchowski W., Gozdowski D., Baczewska A.H., 2011. Comparison of four bioindication methods for assessing the degree of environmental lead and cadmium pollution. J. Hazard. Mater. 197, 109-118.

Giordano S., Adamo P., Sorbo S., Vingiani S., 2005. Atmospheric trace metal pollution in the Naples urban area based on results from moss and lichen bags. Environ. Pollut. 136, 431-442.

Goodarzi F., Sanei H., Labonté M., Duncan W.F., 2002. Sources of lead and zinc associated with metal smelting activities in the Trail area, British Columbia, Canada. J. Environ. Monitor. 4, 400-407.

Kupiainen K., Tervahattu H., 2004. The effect of traction sanding on urban suspended particles in Finland. Environ. Monit. Assess. 93, 287-300.

Muskett C.J., 1976. A survey of airborne heavy metals in a city environment using biological indicators. Environ. Health. 267–269.

Ratcliffe J.M., 1975. An evaluation of the use of biological indicators in an atmospheric lead survey. Atmos. Environ. 9, 623-629.

Szczepaniak K., Astel A., Simeonov V., Tsakovski S., Biziuk M., Bode P., Przyjazny A., 2007. Comparison of dry and living Sphagnum palustre moss samples in determining their biocumulative capability as biomonitoring tools. J. Environ. Sci. Health, Part A. 42(8), 1101-1115.

Vasconcelos M.T.S.D., Tavares H.M.F., 1998. Atmospheric metal pollution (Cr, Cu, Fe, Mn, Ni, Pb and Zn) in Oporto city derived from results for low-volume aerosol samplers and for the moss Sphagnum auriculatum bioindicator. Sci. Total Environ. 212, 11–20.

Vingiani S., De Nicola F., Purvis W.O., Concha-Graña E., Muniategui-Lorenzo S., López-Mahía P., Giordano S., Adamo S., 2015. Active Biomonitoring of heavy metals and PAHs with mosses and lichens: a case study in the cities of Naples and London. Water Air Soil Pollut. 226(8), 240.

Vuković G., Urošević M.A., Pergal M., Janković M., Goryainova Z., Tomašević M., Popović A., 2015. Residential heating contribution to level of air pollutants (PAHs, major, trace, and rare earth elements): a moss bag case study. Environ. Sci. Pollut. Res. Int. 22(23), 18956-66.

Yurukova L., Ganeva A., 1997. Active biomonitoring of atmospheric element deposition with Sphagnum species around a copper smelter in Bulgaria. J. Appl. Bot. 71, 14-20.

Chapter 2

Submitted to Environmental Pollution

Submission date 23/02/2016

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.

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. MATERIALS 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/).

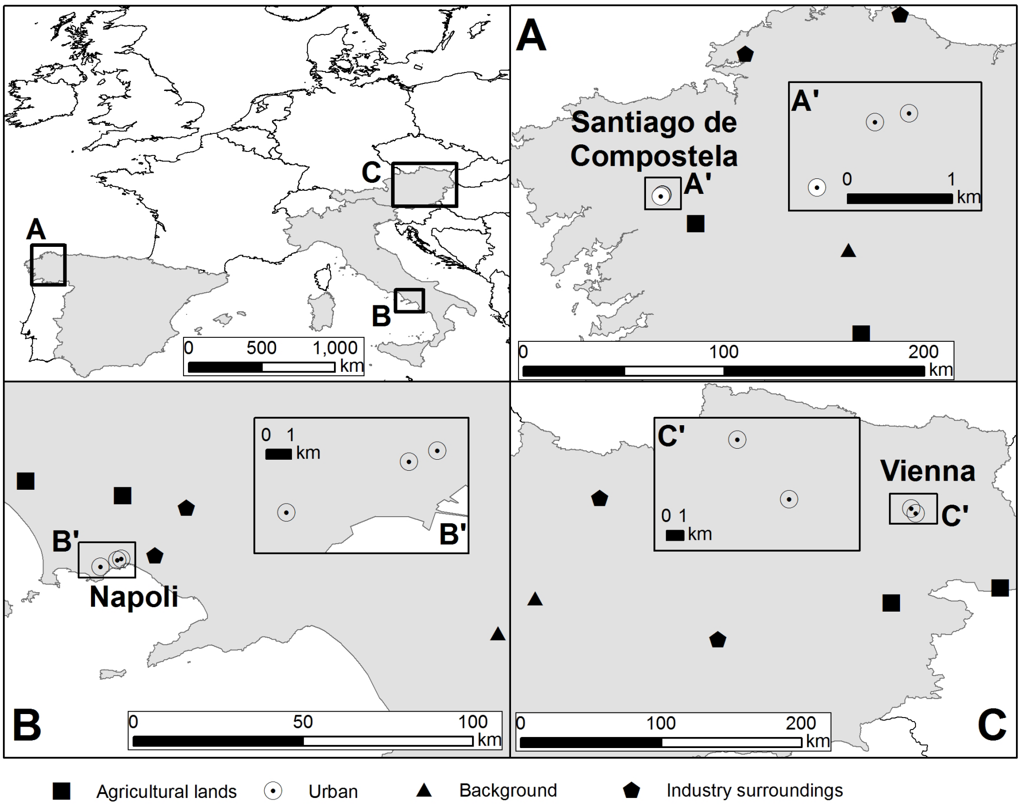

Figure 1. Maps showing the seven stations distributed in industrial, urban, agricultural and background scenarios in Spain (A), Italy (B) and Austria (C).

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.


a) b) c)

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

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 palce 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 weight effect, 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 accumulations occurred comparing 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).

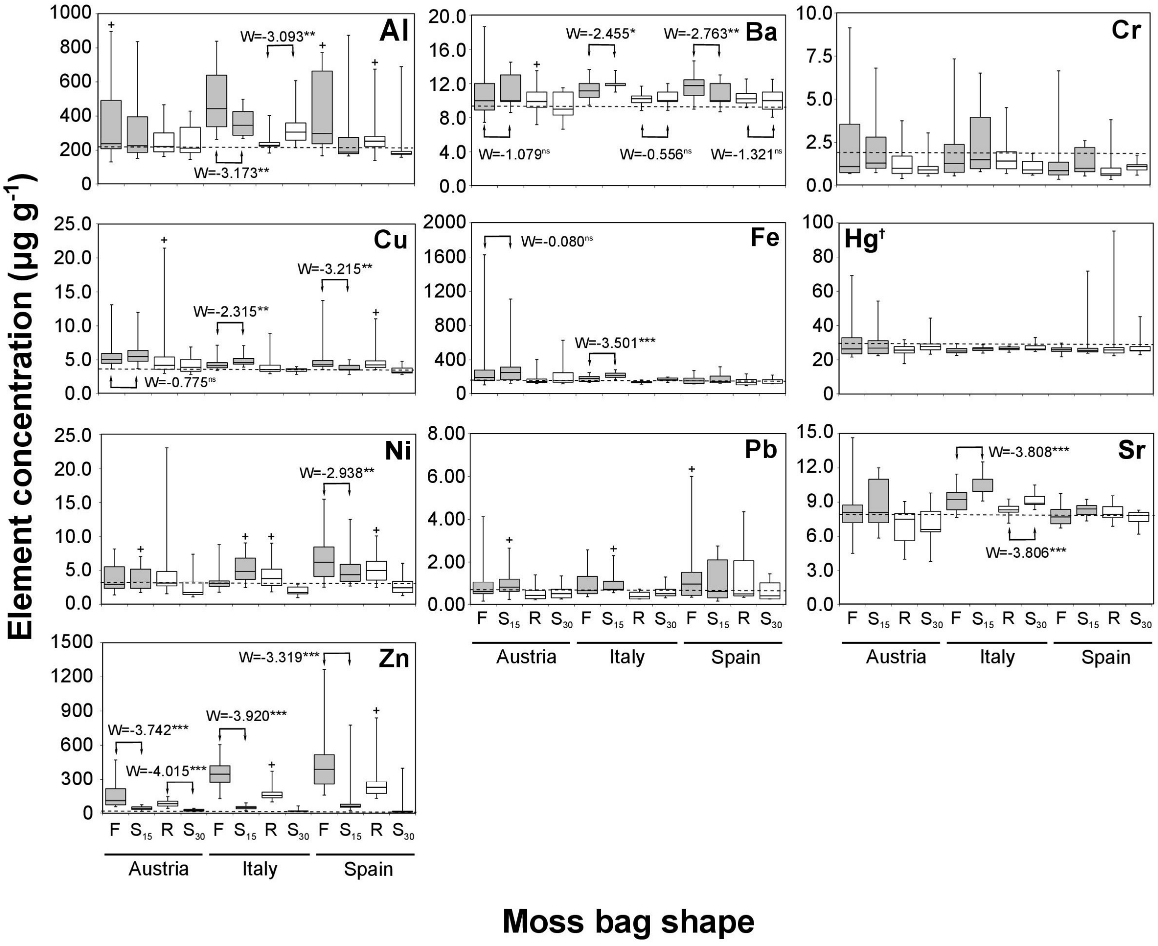

Figure 3 - Box-plots of element concentrations (mg/kg) in the moss exposed in bags of different shape in Austria, Italy and Spain (flat= F *vs* S_{15} Mossphere, grey; rounded=R *vs* S_{30} Mossphere, white). The dashed line represents the LOQ_T. BOX: inside band= median; extremities=1st and 3rd quartiles;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

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

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).

3.2.2 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 (Fig. 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 (Tab 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 Fig. 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.

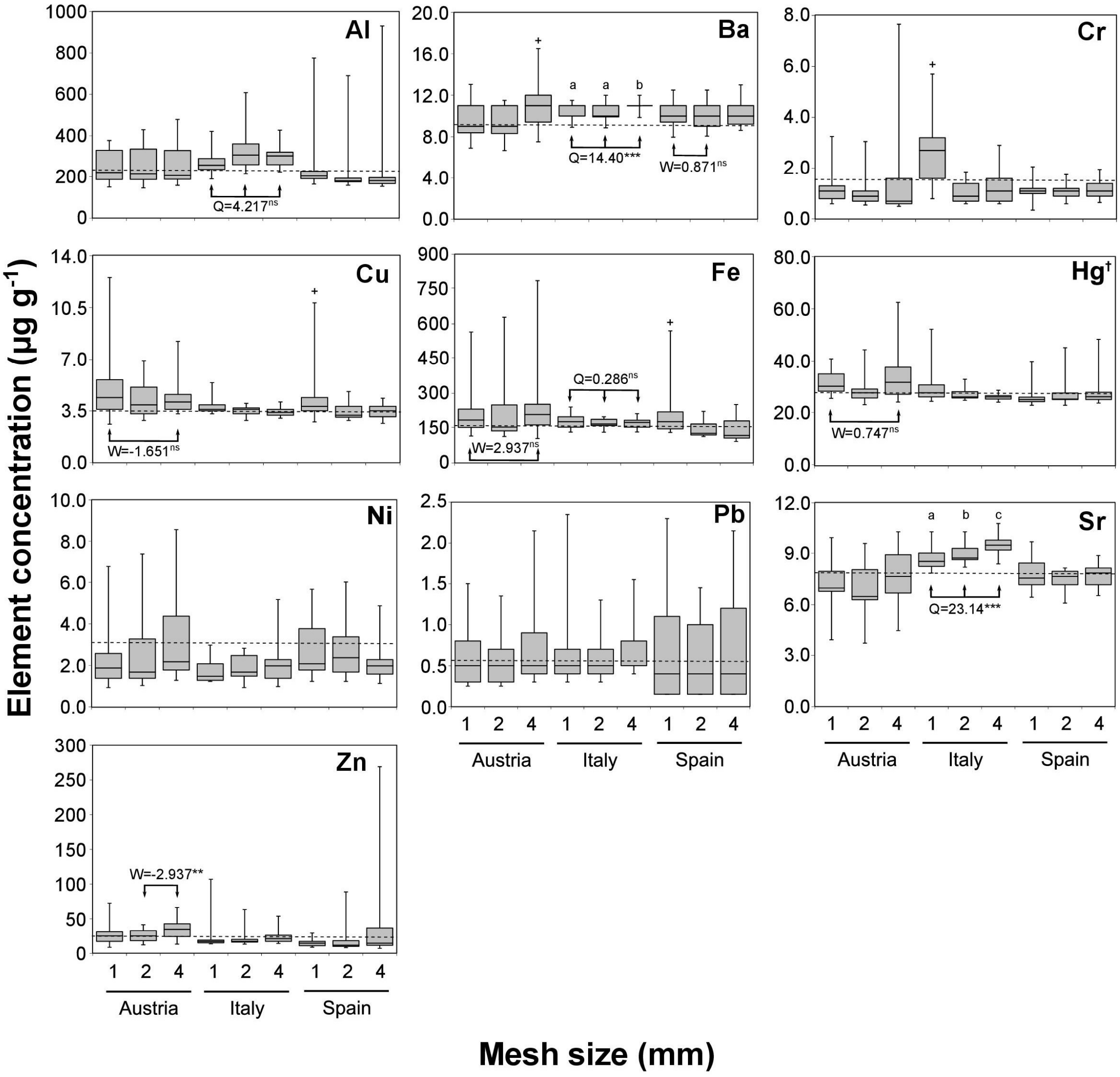

Figure 4 - Box-plots of element concentrations (mg/kg) in the moss exposed in Mosspheres with different mesh sizes (1, 2, 4 mm) in Austria, Italy and Spain. The grey dashed line represents the LOQ$_T$. BOX: inside band= median; extremities=1st and 3rd quartiles; whiskers= MIN and MAX. W, Q are the values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

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

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” (Fig. 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.

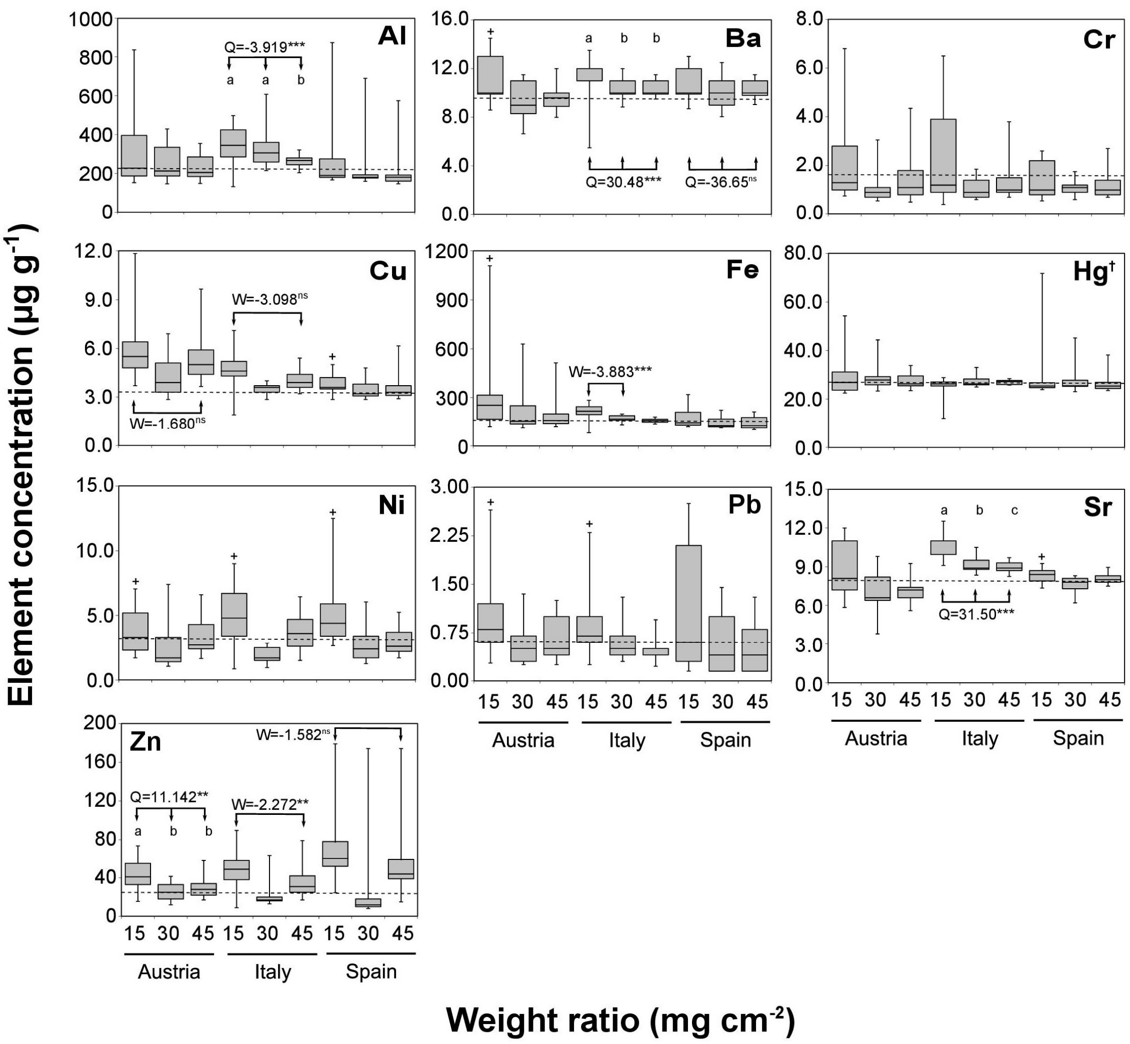

Figure 5 - Box-plots of the element concentrations (mg/kg) in the moss exposed in Mosspheres prepared with different moss weight/sphere surface ratio (W15, W30 and W45) in Austria, Italy and Spain, see text for specifications. The dashed line represents the LOQ_T. BOX: inside band= median; extremities=1st 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.

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

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 (Fig. 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).

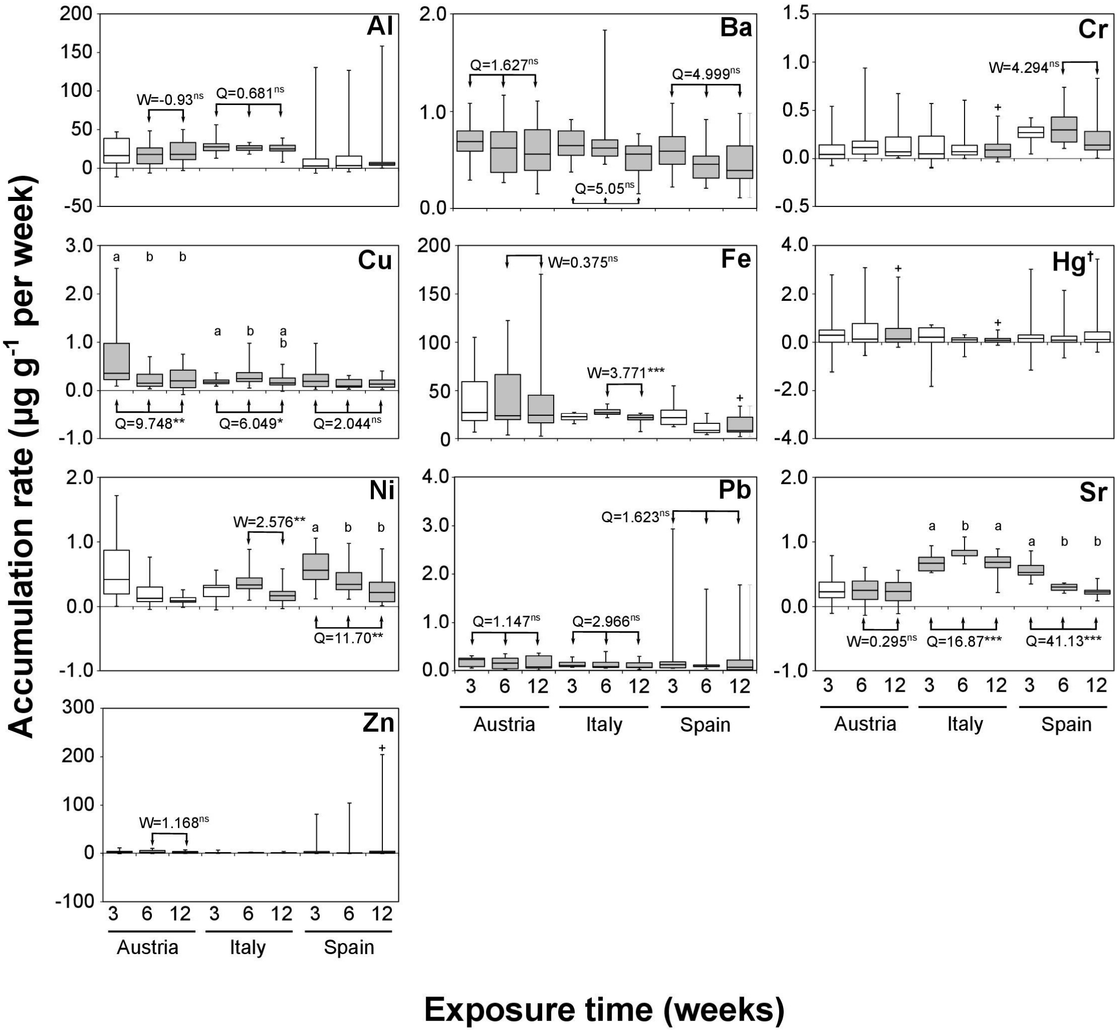

Figure 6 - Box-plots of the element concentrations (mg/kg) in the moss exposed in Mosspheres for 3, 6 and 12 weeks in Austria, Italy and Spain, see text for specifications. The grey dashed line represents the LOQ$_T$. BOX: inside band= median; extremities=1st and 3rd quartiles; whiskers= MIN and MAX. 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.

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

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. 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).

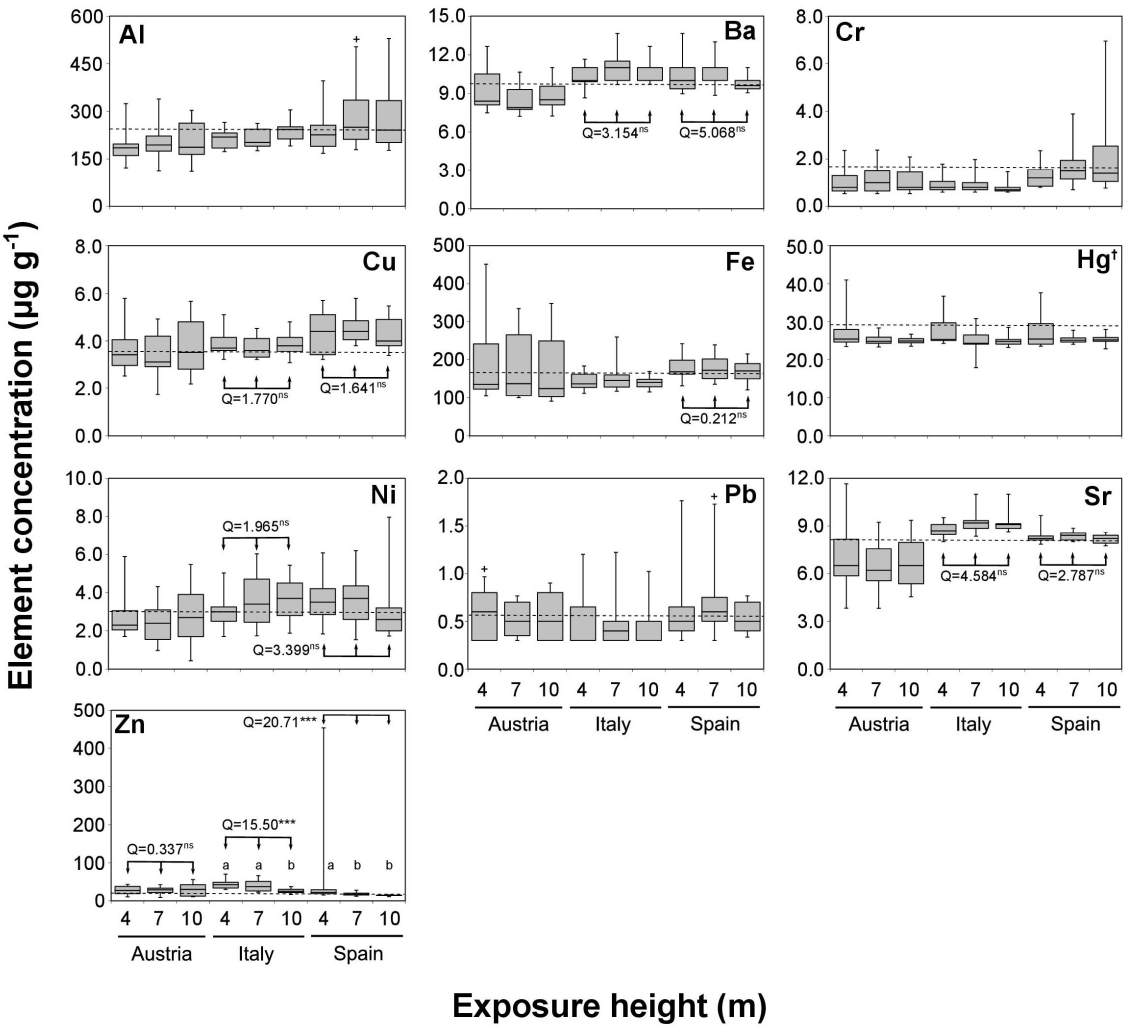

Figure 7 - Box-plots of element concentrations (mg/kg) in the moss exposed in Mosspheres at 4, 7 and 10 m above the ground in Austria, Italy and Spain. The grey dashed line represents the LOQT. BOX: inside band= median; extremities=1st and 3rd quartiles; whiskers= MIN and MAX. Q are the values of Friedman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

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

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 splenden*s 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; Gonzalez 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 Gonzalez 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.

ACKNOWLEDGMENTS

This study received financial support from FP7-ENV.2011.3.1.9-1 MOSSclone project of the European Union in the Seventh Framework Program (FP7) for Research and Technological Development. Thanks are also due to: Stadt Wien - Magistratsabteilung 33 & Magistratsabteilung 49, Stadt Linz – Gebäudemanagement und Tiefbau, Stadtwerke Leoben, Österreichische Bundesforste (ÖBF), Familie Schrammel, Michael Andert, Veronika Fasching.

Bibliography

Adamo P., Giordano S., Vingiani S., Cobianchi R.C., Violante P., 2003. Trace element accumulation by moss and lichen exposed in bags in the city of Naples (Italy). Environ. Pollut. 122, 91–103.

Adamo P., Giordano S., Sforza A., Bargagli R., 2011. Implementation of airborne trace element monitoring with devitalised transplants of *Hypnum cupressiforme* Hedw.: assessment of temporal trends and element contribution by vehicular traffic in Naples city. Environ Pollut. 159, 1620–8.

Aničić, M., Tasić, M., Frontasyeva, M.V., Tomašević, M., Rajšić, S., Strelkova, L.P., Popović, A., Steinnes, E., 2008. Active biomonitoring with wet and dry moss: A case study in an urban area. Environ. Chem. Lett. 7, 55–60.

Aničić M, Tomašević M, Tasić M, Rajšić S, Popović A, Frontasyeva MV, et al. 2009. Monitoring of trace element atmospheric deposition using dry and wet moss bags: accumulation capacity versus exposure time. J. Hazard. Mater. 171, 182–8.

Archibold O. W., 1985. The metal content of wind-blown dust from uranium tailings in Northern Saskatchewan. Water, Air, Soil Pollut. 24, 63–76.

Ares A., Aboal J.R., Carballeira A., Giordano S., Adamo P., Fernandez J.A., 2012. Moss bag biomonitoring: a methodological review. Sci. Total Environ. 158, 432–143.

Ares A., Fernández J.A., Carballeira A., Aboal J.R., 2014. Towards the methodological optimization of the moss bag technique in terms of contaminants concentrations and replicability values. Atmos. Environ. 94, 496–507.

Ares A., Aboal J.R., Carballeira A., Fernández J.A., 2015. Do moss bags containing devitalized Sphagnum denticulatum reflect heavy metal concentrations in bulk deposition? Ecol. Indic. 50, 90-98.

Basile A., Sorbo S., Aprile G., Conte B., Cobianchi R.C., 2008. Comparison of the heavy metal bioaccumulation capacity of an epiphytic moss and an epiphytic lichen. Environ Pollut. 151, 401–7.

Basile A., Sorbo S., Aprile G., Conte B., Castaldo Cobianchi R., Pisani T., Loppi S., 2009. Heavy metal deposition in the Italian ''triangle of death'' determined with the moss *Scorpiurum circinatum.* Environ. Pollut. 157, 2255–2260.

Beike A.K., Spagnuolo V., Lüth V., Steinhart F., Ramos-Gomez J., Krebs M., Adamo P., Rey-Asensio A.I., Giordano S., Decker E.L., Reski R., 2015. Clonal *in vitro* propagation of peat mosses (*Sphagnum* L.) as novel Green resources for basic and applied research. PTOC 120, 1037–1049.

Beelen R.,et al., 2014. Effects of long-term exposure to air pollution on natural-cause mortality: an analysis of 22 European cohorts within the multicentre ESCAPE project. Lancet. 383, 785-795.

Boquete T., Fernández J.A., Carballeira A., Aboal J.R., 2013. Assessing the tolerance of the terrestrial moss *Pseudoscleropodium purum* to high levels of atmospheric heavy metals: a reciprocal transplant study. Sci. Total Environ. 461-462, 552–559.

Concha-Gran E., Muniategui-Lorenzo S., De Nicola F., Aboal J.R., Rey-Asensio A.I., Giordano S., Reski R., Lopez-Mahia P., Prada-Rodriguez D., 2015. Matrix solid phase dispersión method for determination of polycyclic aromatic hydrocarbons in moss. J. Chromatogr. A 1406, 19–26.

Couto J.A., Aboal J.R., Fernández J.A., Carballeira A., 2004. A new method for testing the sensitivity of active biomonitoring: an example of its application to a terrestrial moss. Chemosphere 57, 303–308.

De Nicola F., Murena F., Costagliola M.A., Alfani A., Baldantoni D., Prati M.V., Sessa L., Spagnuolo V., Giordano S., 2013. A multi-approach monitoring of particulate matter, metals and PAHs in an urban street canyon. Environ. Sci. Pollut. Res. 20, 4969–4979.

Gailey F.A.Y. Lloyd O.L., 1986a. Atmospheric metal pollution monitored by spherical moss bags: a case study of Armadale. Environ. Health Perspect. 68, 187–96.

Gailey F.A.Y, Lloyd O.L., 1986b. Methodological investigations into low technology monitoring of atmospheric metal pollution: part I. The effects of sampler size on metal concentrations. Environ. Pollut. Ser. B Chem. Phys. 12:41–59.

Gailey F.A.Y., Lloyd O.L., 1986c. Methodological investigations into low technology monitoring of atmospheric metal pollution: part II. The effects of length of exposure on metal concentrations. Environ. Pollut. Ser. B Chem. Phys. 12:61–74.

Gailey F.A.Y., Lloyd O.L., 1986d. Methodological investigations into low technology monitoring of atmospheric metal pollution: part III. The degree of replicability of the metal concentrations. Environ. Pollut. Ser. B Chem Phys. 12, 85–109.

Giordano S., Adamo P., Monaci F., Pittao E., Tretiach M., Bargagli R., 2009. Bags with oven-dried moss for the active monitoring of airborne trace elements in urban areas c Environ. Pollut. 157, 2798–2805

Giordano S., Adamo P., Spagnuolo V., Tretiach M., Bargagli R., 2013. Accumulation of airborne trace elements in mosses, lichens and synthetic materials exposed at urban monitoring stations: Towards a harmonisation of the moss-bag technique. Chemosphere 90, 292–299.

Gonzalez A.G., Pokrovsky O.S., Beike A.K., Reski R., Di Palma A., Adamo P., Giordano S., Fernandez J.A., 2016a. Metal and proton adsorption capacities of natural and cloned *Sphagnum* mosses. J. Colloid Interface Sci. 461, 326–334.

Gonzalez A.G., Jimenez-Villacorta F., Beike A.K., Reski R., Adamo P., Pokrovsky O.S., 2016b. Chemical and structural characterization of copper adsorbed on mosses (Bryophyta). J. Hazard. Mater. 308, 343–354.

Goodman G.T., Roberts T.M., 1971. Plants and soils as indicators of metals in the air. Nature (Lond.), 231–287.

Harmens H., Mills G., Hayes F., Norris D.A., Sharps K., 2015. Twenty-eight years of ICP Vegetation: an overview of its activities. Ann. Bot. 5, 31–43.

Laschober C., Limbeck A., Rendl J., Puxbaum H., 2004. Particulate emissions from on-road vehicles in the Kaisermühlen-Tunnel (Vienna, Austria). Atmos. Environ. 38, 2187–2195.

Little P., Martin M.H., 1974. Biological monitoring of heavy metal pollution. Environ. Pollut. 6, 1-19.

Napier F., D’Arcy B., Jefferies C., 2008. A review of vehicle related metals and polycyclic aromatic hydrocarbons in the UK environment. Desalination. 226, 143–150.

Ratcliffe J.M., 1975. An evaluation of the use of biological indicators in an atmospheric lead survey. Atmos. Environ. 9, 623–629.

Rivera M., Zechmeister H., Medina-Ramón M., Basagaña X., Foraster M., Bouso L., Moreno T., Solanas P., Ramos R., Köllensperger G., Deltell A., Vizcaya D., Künzli N., 2011. Monitoring of heavy metal concentrations in home outdoor air using moss bags. Environ. Pollut. 159, 954–62.

Steinnes E., Rühling Å., Lippo H., Makinen A., 1997. Reference materials for large-scale metal deposition surveys. Accred. Qual. Assur. 2, 243–249.

Strachan W.M.J., Glooschenko W.A., 1988. Moss bags as monitors of organic contamination in the atmosphere. Bull. Environ. Contam. Toxicol. 40, 447–50.

Tavares H.M.C.F., Vasconcelos M.T.S.D., 1996. Comparison of lead levels collected by *Sphagnum auriculatum* and by a low-volume aerosol sampler in the urban atmosphere of Oporto. Toxicol. Environ. Chem. 54, 195–209.

Temple P.J., McLaughlin D.L., Linzon S.N., Wills R., 1981. Moss bags as monitors of atmospheric deposition. J. Air Pollut. Control. Assoc. 31, 668–70.

Thorpe A., Harrison R.M., 2008. Sources and properties of non-exhaust particulate matter from road traffic: a review. Sci. Total Environ. 400, 270–282.

Vasconcelos M.T.S.D., Tavares H.M.F., 1998. Atmospheric metal pollution (Cr, Cu, Fe, Mn, Ni, Pb and Zn) in Oporto city derived from results for low-volume aerosol samplers and for the moss *Sphagnum auriculatum* bioindicator. Sci. Total. Environ. 212, 11–20.

Vukovic G., Anicic Urosevic M., Razumenic I., Goryainova Z., Frontasyeva M., Tomasevic M., Popovic A., 2013. Active moss biomonitoring of small-scale spatial distribution of airborne major and trace elements in the Belgrade urban area. Environ. Sci. Pollut. Res. 20, 5461–5470.

Zechmeister H.G., Hohenwallner D., Riss A., Hanus-Illnar A., 2005. Estimation of element deposition deriving from road traffic sources by mosses. Environ. Pollut. 138, 238–249

Zechmeister H.G., Hagendorfer H., Hohenwallner D., Hanus-Illnar A., Riss A., 2006. Analyses of platinum group elements in mosses as indicators of road traffic emissions in Austria. Atmos. Environ. 40, 7720–7732.

Sitography

- http://www.zamg.ac.at/
- http://www.sito.regione.campania.it/
- http://www.meteogalicia.es/

Chapter 3

Science of the Total Environment 541 (2016) 1410–1419

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 post-exposure contents were higher than the

pre-exposure 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.

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.

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

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.

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**

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.

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**

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.

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

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.

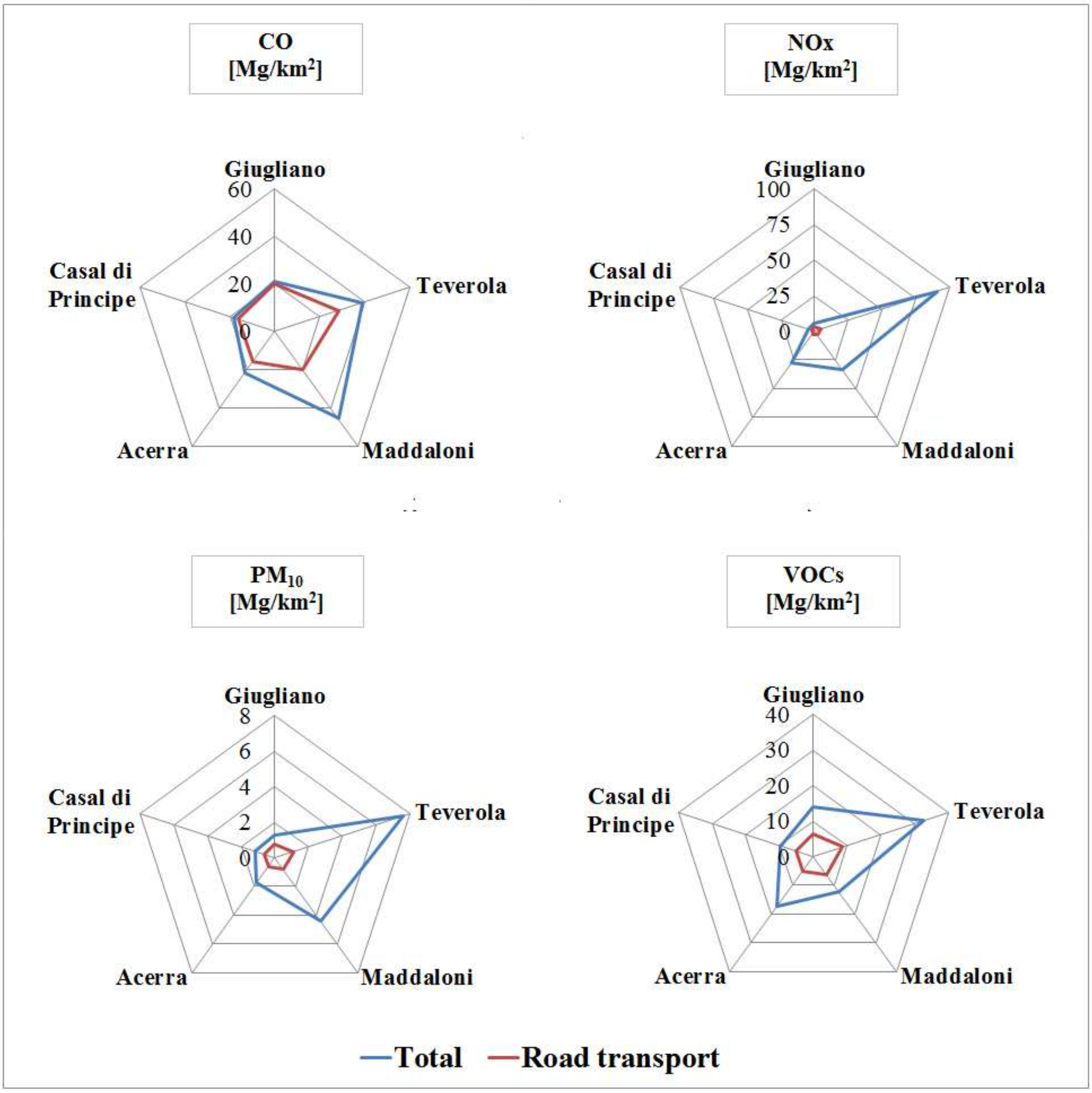

Figure 2. Total emissions and road transport contribution of each examined Municipality for the following pollutants: CO, NO_X, PM_{10},VOCs.

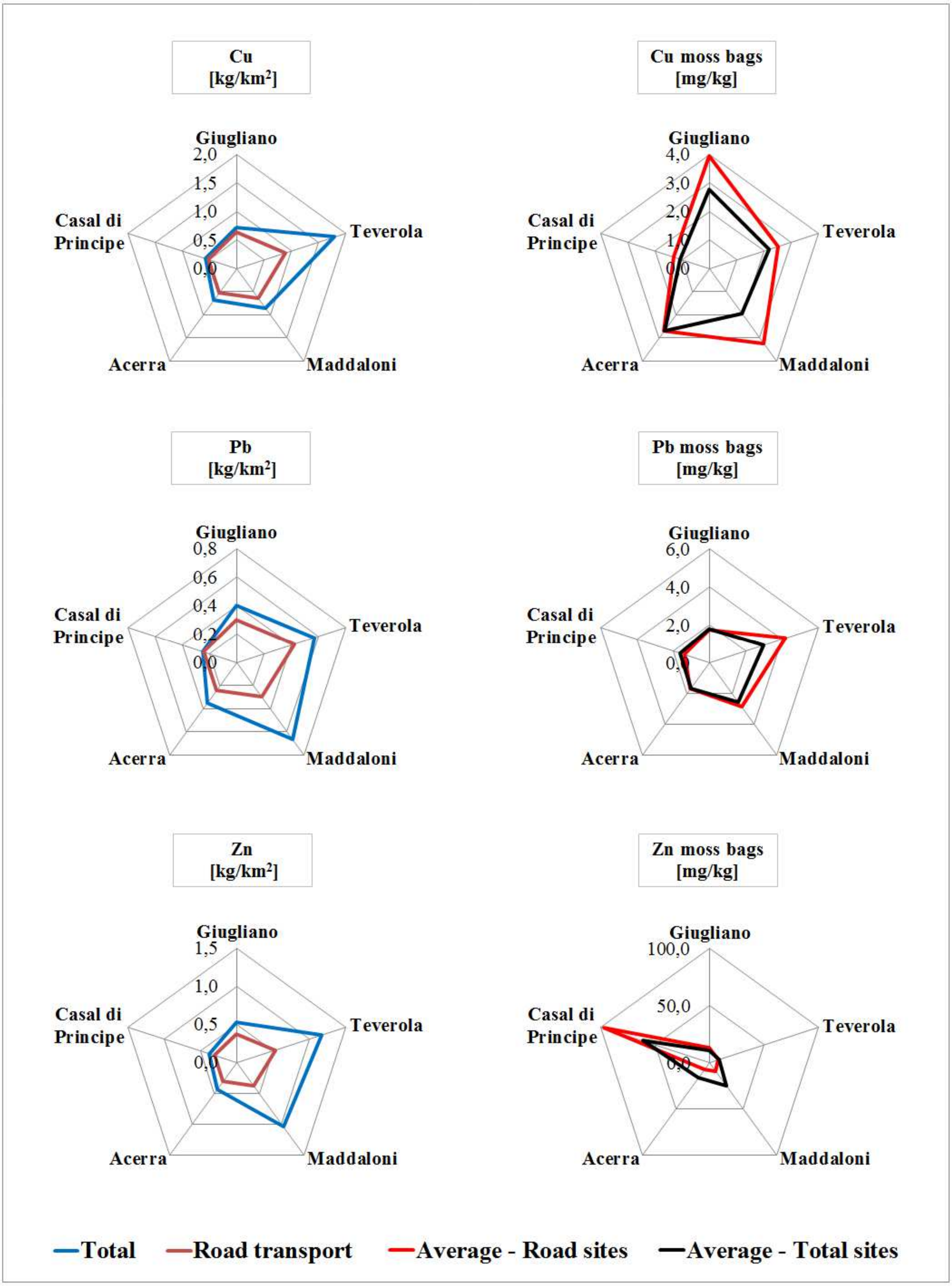

Figure 3. Total emissions and road transport contribution for Cu, Pb and Zn (left) and moss uptake of the same elements (right) at each Municipality.

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%).

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

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.

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**

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.

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

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.

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

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.

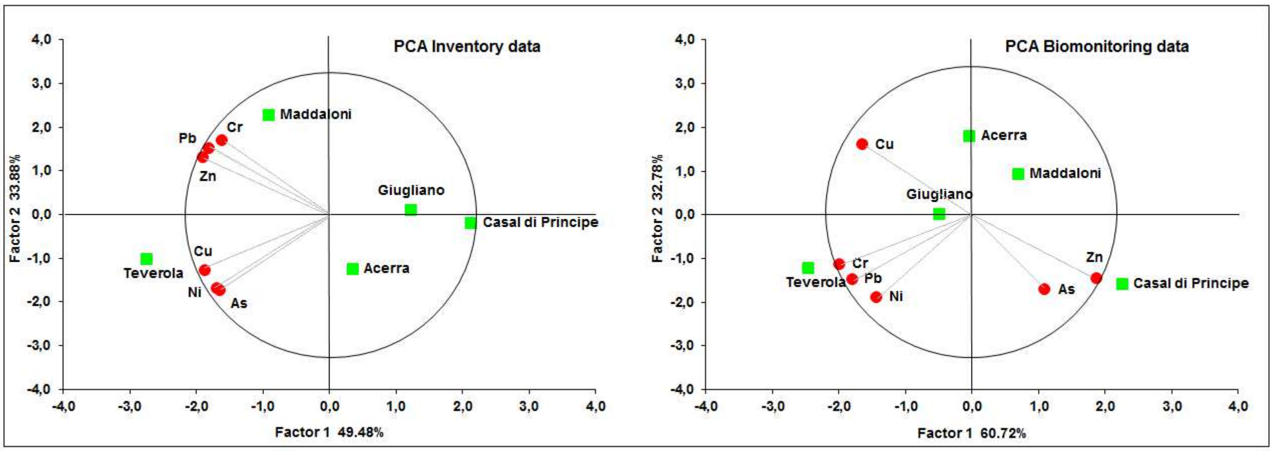

Figure 4. PCA of emission inventory metal data (left) and moss uptake (right) grouped by Municipality.

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.

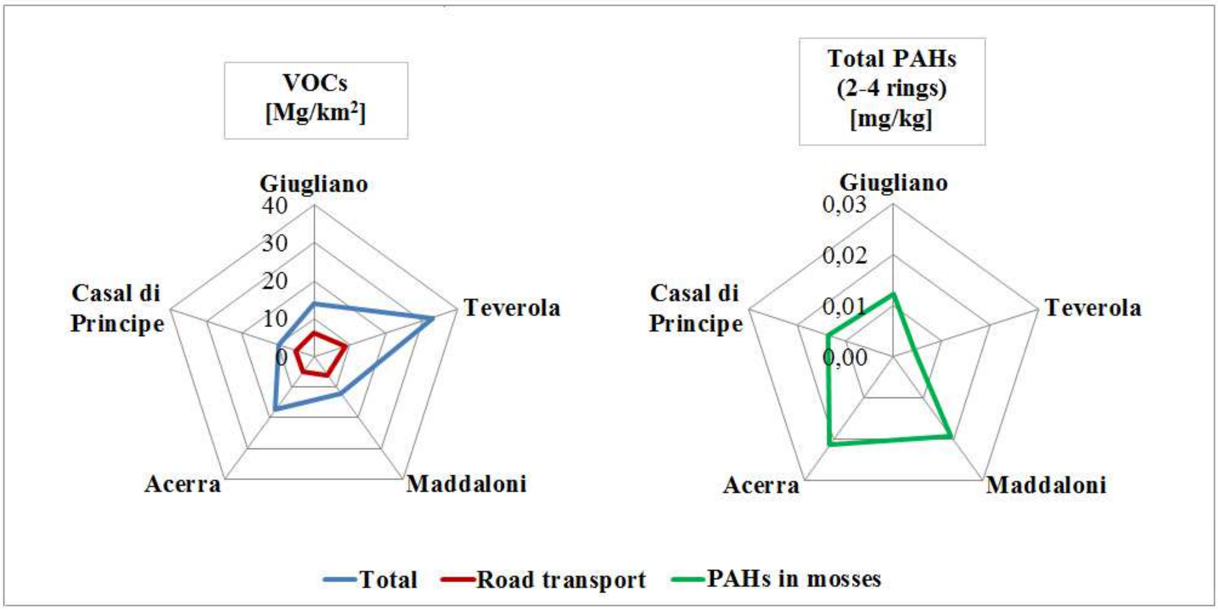

Figure 5. Total emissions and road transport contribution for VOCs (left) and moss uptake of PAHs (2-4 rings) (right) at each Municipality.

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.

ACKNOWLEDGMENT

This study was financed by the EU Commission, Project: LIFE/11/ENV/IT/275 ECOREMED (*Implementation of ecocompatible protocols for agricultural soil remediation in Litorale Domizio-Agro Aversano NIPS*)

References

Adamo P., Bargagli R., Giordano S., Modenesi P., Monaci F., Pittao E., Spagnuolo V., Tretiach M., 2008. Natural and pre-treatments induced variability in the chemical composition and morphology of lichens and mosses selected for active monitoring of airborne elements. Environ. Pollut. 152, 11–19.

Al-Fatlawi SMG, Al-Alwani M., 2012. Heavy metal pollution of roadside dust samples with different traffic volumes at Hilla city. Iraqi J. Mech. Mater. Eng. 12(4), 660–672.

Ares A, Aboal JR, Carballeira A, Giordano S, Adamo P, Fernandez JA, 2012. Moss bag biomonitoring: a methodological review. Sci. Total Environ. 432, 143–158.

Automobile Club d’Italia, 2011. Annuario Statistico - Automobilismo e Trasporti. Ufficio Statistica, Roma, ACI 2011. Available at http://www.aci.it/laci/studi-e-ricerche/dati-e-statistiche/annuario-statistico/annuario-statistico-2011.html.

Bellasio R, Bianconi R, Corda G, Cucca P., 2007. Emission inventory for the road transport sector in Sardinia. Atmos.Environ. 41, 677-691.

Bove MA, Ayuso RA, De Vivo B, Lima A, Albanese S., 2011. Geochemical and isotopic study of soils and waters from an Italian contaminated site: Agro Aversano (Campania). J. Geochem. Explor. 109(1–3), 38–50.

Capra GF, Coppola E, Odierna P, Grilli E, Vacca S, Buondonno A., 2014. Occurrence and distribution of key potentially toxic elements (PTEs) in agricultural soils: A paradigmatic case study in an area affected by illegal landfills. J. Geochem. Explor. 145, 169–180.

CORINAIR, 1988. European Inventory of emissions of pollutants into the atmosphere. Commission of the EC–CORINAIR project, DG XI.

Corniello A., Ducci D., 2014. Hydrogeochemical characterization of the main aquifer of the “Litorale Domizio-Agro Aversano NIPS” (Campania—southern Italy). J Geochem Explor. 137, 1-10.

De Nicola F., Murena F., Costagliola M.A., Alfani A., Baldantoni D., Prati M.V., Sessa L., Spagnuolo V., Giordano S., 2013. A multi-approach monitoring of particulate matter, metals and PAHs in an urban street canyon. Environ. Sci. Pollut. Res. Int. 20, 4969–4979.

EU Air Quality Directive, 2008. Directive 2008/50/EC of the European Parliament and of the Council of 21 May 2008 on ambient air quality and cleaner air for Europe. EU, 2008. Official J., pp. 1–44 (11.062008, L152).

European Communities, 1992. Regulation 2081/92, Official Journal of European Communities, L208.

European Communities 1996. Regulation 1107/96, Official Journal of European Communities, L148.

European Environment Agency, 1998. Guidance report on preliminary assessment under EC air quality directives. Technical Report No. 11.

European Environment Agency, 2009. EMEP/CORINAIR Emission Inventory Guidebook. Technical Report No. 9.

Frati L., Brunialti G., Loppi S., 2005. Problems related to lichen transplants to monitor trace element deposition in repeated surveys: a case study from central Italy. J. Atmos. Chem. 52, 221–230.

Gimelli A., Luongo A., Amoresano A., 2012. Experimental Data and Thermodynamic Analysis of Biomass Steam Power Plant with Two Different Configurations Plant. Int. Rev. Mech. Eng. 6(6), 1109-1116.

Gkatzoflias D., Kouridis C., Ntziachristos L., Samaras Z., 2012. COPERT 4 Computer programme to calculate emissions from road transport. Available at http://www.emisia.com/copert/.

Grezzi G., Ayuso R.A., De Vivo B., Lima A., Albanese S., 2011. Lead isotopes in soils and groundwaters as tracers of the impact of human activities on the surface environment: the Domizio-Flegreo Littoral (Italy) case study. J. Geochem. Explor. 109(1–3), 51–58.

Intergovernmental Panel on Climate Change, 1997. IPCC guidelines for national greenhouse gas inventories. Available at http://www.ipcc-nggip.iges.or.jp/public/gl/invs1.html.

Iodice P., Senatore A., 2013a. Road transport emission inventory in a regional area by using experimental two-wheelers emission factors. Lecture Notes in Engineering and Computer Sciences, World Congress on Engineering, London, 681-685.

Iodice P., Senatore A., 2013b. Evaluation of Dispersion Models for Predicting Carbon Monoxide Concentrations from Motor Vehicles in a Metropolitan Area. Int Rev Model Simul. 6(6), 1928-1932.

Iodice P., Senatore A., 2014a. Experimental-analytical investigation to estimate an emission inventory from road transport sector. IAENG Transactions on Engineering Sciences - Special Issue of the International MultiConference of Engineers and Computer Scientists, IMECS 2013 and World Congress on Engineering, WCE 2013, 141-149

Iodice P., Senatore A., 2014b. Cold start emissions of a motorcycle using ethanol-gasoline blended fuels. Energy Procedia. 45, 809–818.

Iodice P, Senatore A. Appraisal of pollutant emissions and air quality state in a critical Italian region: methods and results. Environmental Progress & Sustainable Energy. In press 2015a. DOI: 10.1002/ep

Iodice P, Senatore A., 2015b. Exhaust emissions of new high-performance motorcycles in hot and cold conditions. Int. J. Environ. Sci. Technol.12, 3133-3144.

Istituto Superiore per la Protezione e la Ricerca Ambientale, 2010. Trasporto su strada – Inventario nazionale delle emissioni e disaggregazione provinciale. ISPRA 2010, Rapporti 124. Available at http://www.isprambiente.gov.it/it/pubblicazioni/rapporti/trasporto-su-strada-inventario-nazionale-delle.

Kabata-Pendias A. Trace elements in Soils and Plants. 4th Edition. CRC Press, Boca Raton, pp. 534.

Mellios G, Aalst RV, Samaras Z., 2006. Validation of road traffic urban emission inventories by means of concentration data measured at air quality monitoring stations in Europe. Atm. Env. 40(38), 7362-7377.

Ministero dell'Industria, del Commercio e dell'Artigianato, 2011. Bollettino Petrolifero, MICA 2011.

Ministero delle Infrastrutture e dei Trasporti, 2011. Conto Nazionale delle Infrastrutture e dei Trasporti. Available at http://www.mit.gov.it/mit/mop_all.php?p_id=12558.

Ntziachristos L., Samaras Z., 2012. Methodology for the calculation of exhaust emissions. SNAPs 070100-070500, NFRs 1A3bi-iv.-EMEP/EEA Emission inventory guidebook.

Ohara T., Akimoto H., Kurokawa J., Horii N., Yamaji K., Yan X., T. Hayasaka, 2007. An Asian emission inventory of anthropogenic emission sources for the period 1980–2020. Atmos. Chem. Phys.7, 4419–4444.

Pal S.K., Wallis S.G., Arthur S., 2011. Assessment of heavy metals emission from traffic on road surfaces. Cent. Eur. J. Chem. 9(2), 314–319.

Senior K., Mazza A., 2004. Italian ‘‘Triangle of death’’ linked to waste crisis. The Lancet, Oncology. 5, 525–527.

Steinnes E., Rühling Å., Lippo H., Makinen A., 1997. Reference materials for large-scale metal deposition surveys. Accred Qual. Assur. 2, 243–249.

Sutherland R.A., Tack F.M.G., Ziegler A.D., 2012. Road-deposited sediments in an urban environment: a first look at sequentially extracted element loads in grain size fractions. J. Hazard Mater. 225–256, 54–62.

U.S. Environmental Protection Agency, 1999. Compilation of air pollutant emission factors (AP-42) Volume 1: Stationary Point and Area Sources.

U.S. Environment Protection Agency, 2001. Control of emissions of hazardous air pollutants from mobile sources; final rule. Federal Register 40, CFR parts 80 and 86. US Government Printing Office, Washington, DC.

Zhang F., Yan X., Zeng C., Zhang M., Shrestha S., Devkota L.P., Yao T., 2012. Influence of Traffic Activity on Heavy Metal Concentrations of Roadside Farmland Soil in Mountainous Areas. J. Environ. Res. Public Health. 9(5), 1715-1731.

Chapter 4

Chemosphere 149 (2016) 211–218

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.

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.

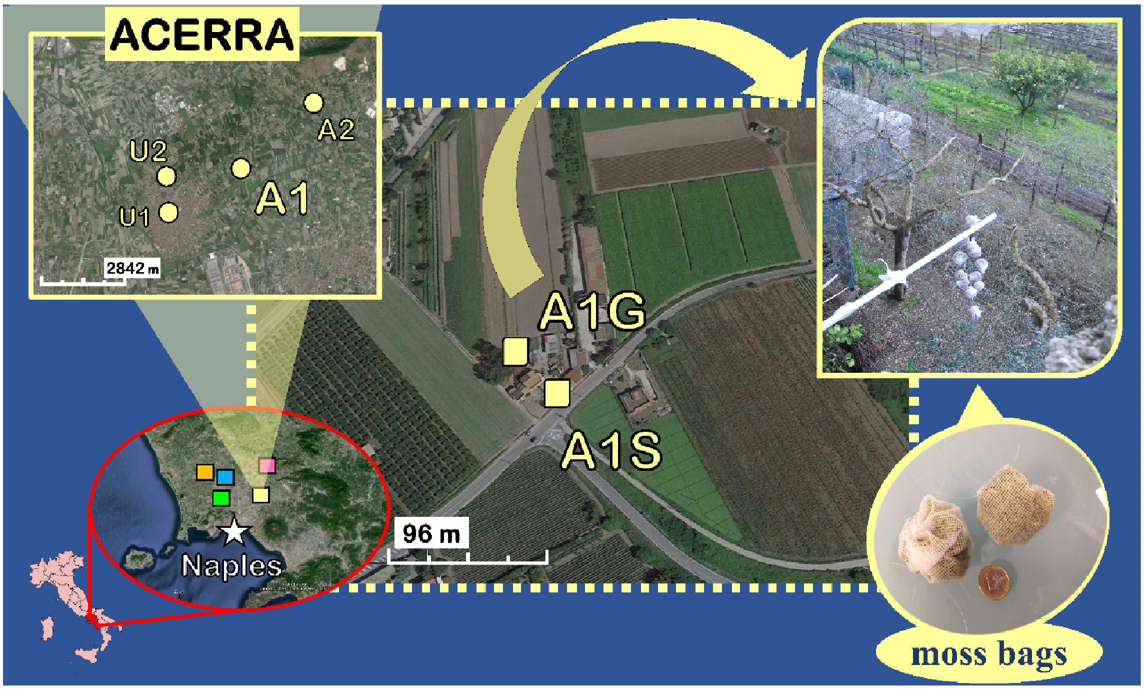

Figure 1. Outline of the moss bag exposure design in the study area with a shoot up on Acerra agricultural (A1 and A2) and urban/residential (U1 and U2) sites. The arrangement of moss bags in green (G) and street (S) exposure points at the A1 site is also shown.

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.

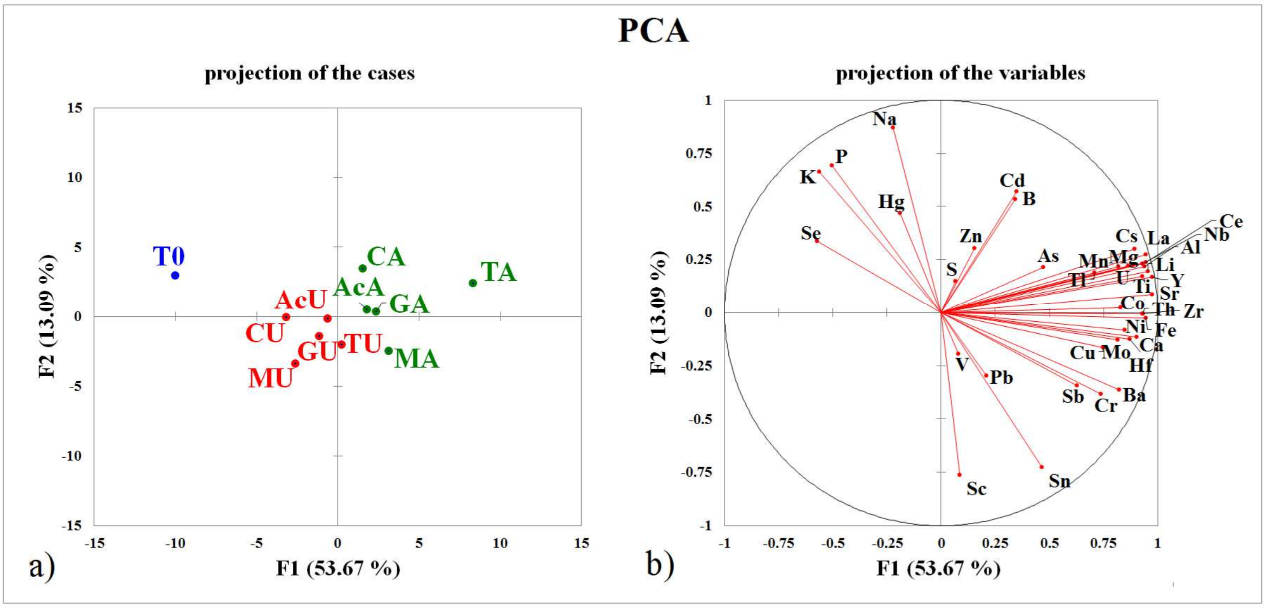

Figure 2. PCA considering agricultural (A) and urban (U) scenarios at each municipality: a) projection of the cases (n = 11); b) projection of the variables (n = 39). See the text for the label codes.

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).

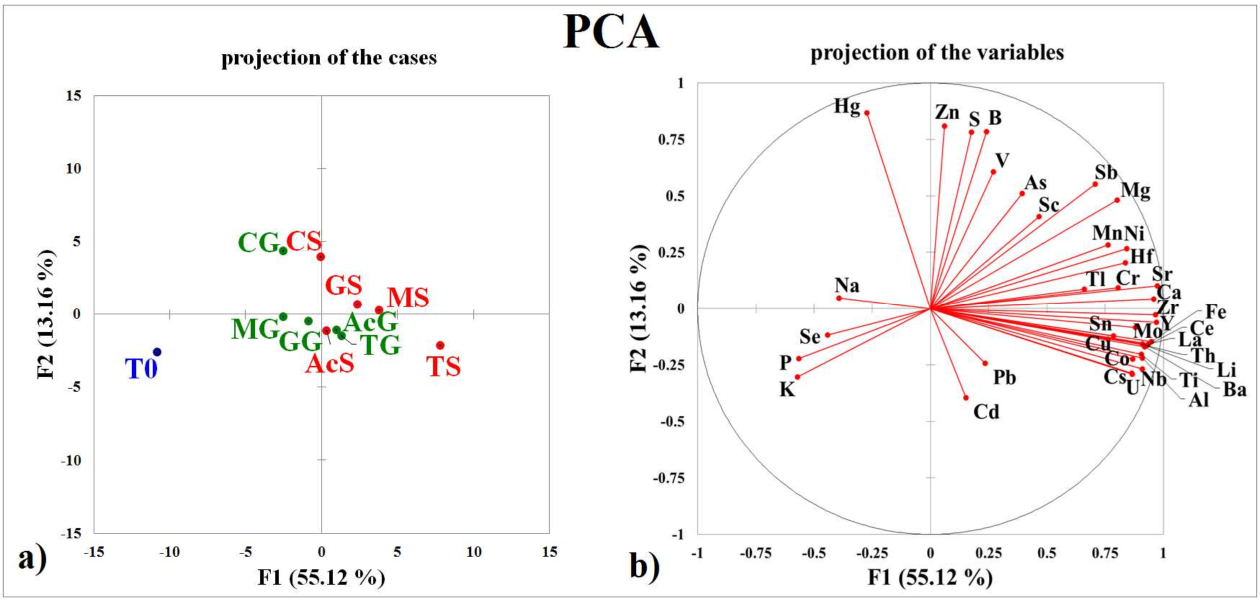

Figure 3. PCA considering green (G) and street (S) sub-scenarios at each municipality: a) projection of the cases (n = 11); b) projection of the variables (n = 39). See text for label codes.

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.

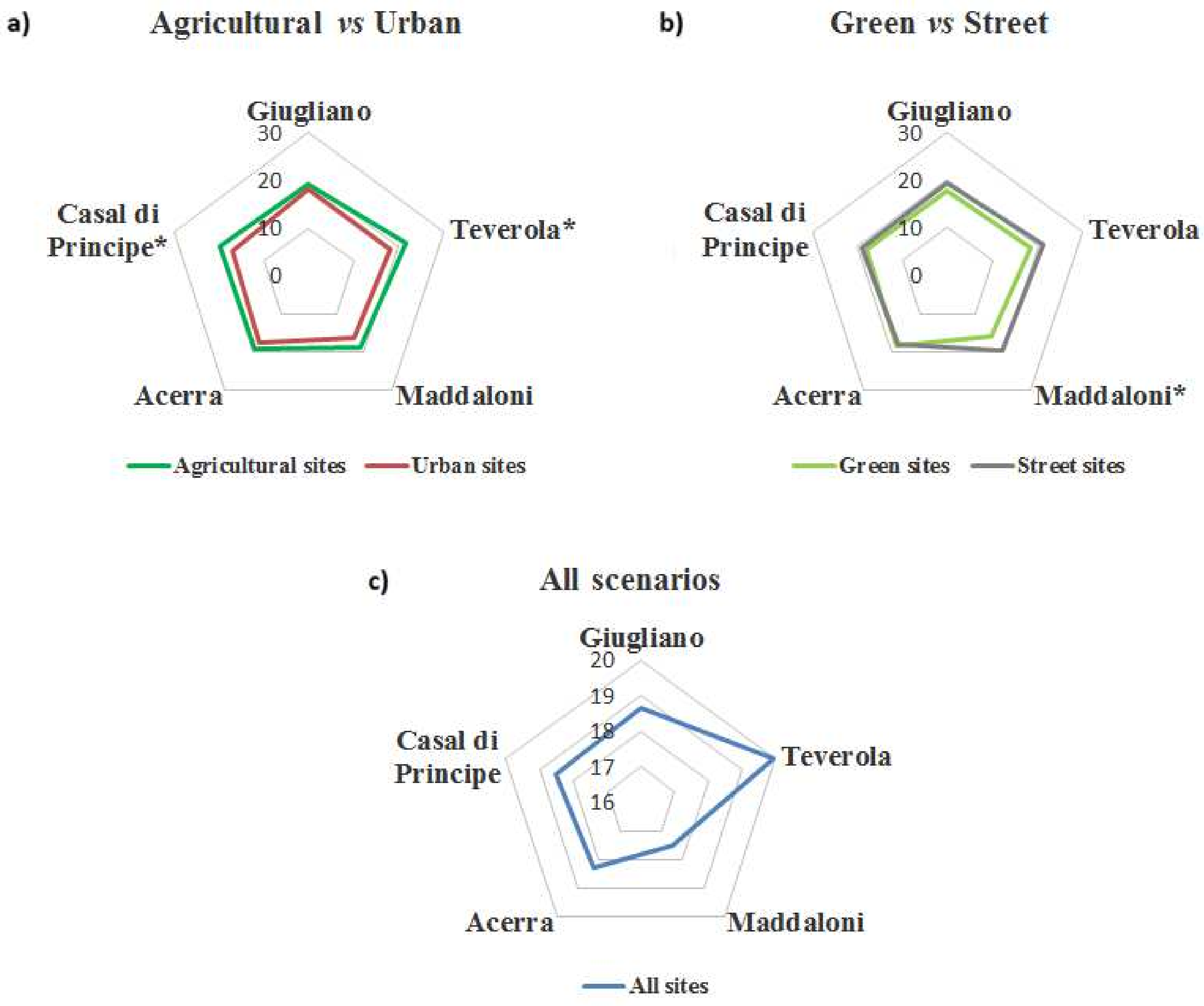

Figure 4. Total element accumulation (sum of the normalized values) observed at each municipality. a) agricultural (A) versus urban (U) sites; b) green (G) versus street (S) sites; c) data from all scenarios. Asterisks indicate that confidence intervals do not overlap.

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).

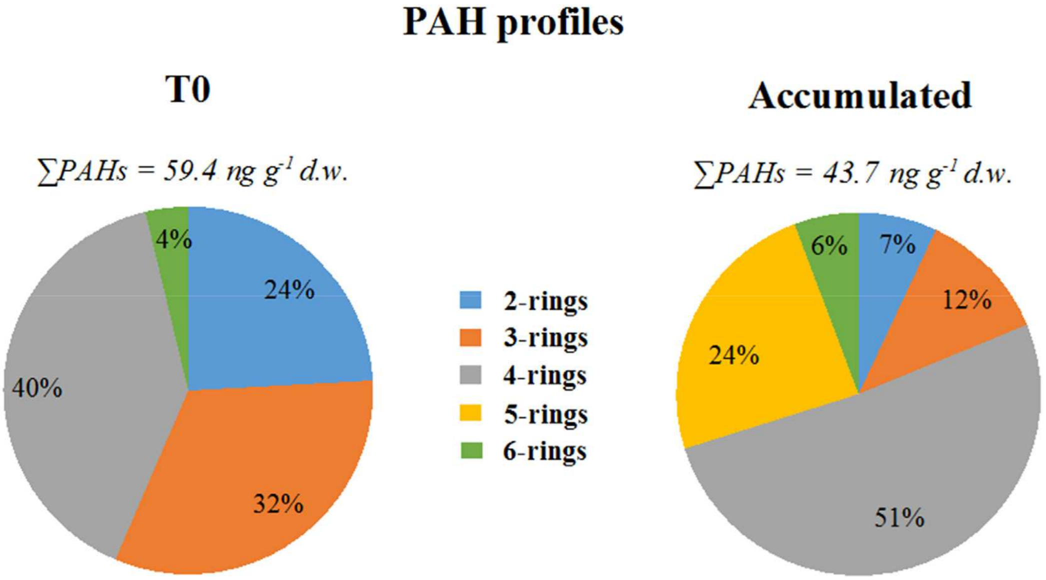

Figure 5. Profiles of PAHs grouped by number of rings, as percentage of total PAHs, in pre-exposure moss (T0) and accumulated in the exposed moss bags (mean over all sites).

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).

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*

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.

Acknowledgments

The present study was financed by the EU Commission, Project: LIFE/11/ENV/IT/275 ECOREMED – "Implementation of eco-compatible protocols for agricultural soil remediation in Litorale Domizio-Agro Aversano NIPS".

References

Adamo P., Crisafulli P., Giordano S., Minganti V., Modenesi P., Monaci F., Pittao E., Tretiach M., Bargagli R., 2007. Lichen and moss bags as monitoring devices in urban areas. Part II: Trace element content in living and dead biomonitors and comparison with synthetic materials. Environ. Pollut. 146, 392-399.

Adamo P., Giordano S., Sforza A., Bargagli R., 2011. Implementation of airborne trace element monitoring with devitalised transplants of Hypnum cupressiforme Hedw.: Assessment of temporal trends and element contribution by vehicular traffic in Naples city. Environ. Pollut. 159, 1620-1628.

Ares A., Aboal J.R., Carballeira A., Giordano S., Adamo P., Fernandez J.A., 2012. Moss bag biomonitoring: a methodological review. Sci. Total Environ. 158, 432-143.

Ares A., Aboal J.R., Carballeira A., Fernandez J.A., 2015. Do moss bags containing devitalized Sphagnum denticulatum reflect heavy metal concentrations in bulk deposition? Ecol. Indic. 50, 90-98.

Augusto S., Maguas C., Matos J., Pereira M.J., Branquinho C., 2010. Lichens as an integrating tool for monitoring PAH atmospheric deposition: A comparison with soil, air and pine needles. Environ. Pollut. 158, 483-489.

Bolton J., 1973. Sources of magnesium for sugar beet, potatoes and wheat grown on an acid sandy soil at Woburn, Beds. J. Agr. Sci. 81, 553-555.

Couto J.A., Aboal J.R., Fernandez J.A., Carballeira A., 2004. A new method for testing the sensitivity of active biomonitoring: an example of its application to a terrestrial moss. Chemosphere 57, 303-308.

De Nicola F., Murena F., Costagliola M.A., Alfani A., Baldantoni D., Prati, M.V. Sessa, L., Spagnuolo V., Giordano S., 2013. A multi-approach monitoring of particulate matter, metals and PAHs in an urban street canyon. Environ. Sci. Pollut. Res. 20, 4969-4979.

di Gennaro A., 2014. Per una storia dell'ecosistema metropolitano di Napoli. In Meridiana 80, Città metropolitana pp. 105-124, Viella SRL.

EU Air Quality Directive, 2008. Directive 2008/50/EC of the European Parliament and of the Council of 21 May 2008 on ambient air quality and cleaner air for Europe. Official J., (11.06.2008, L152).

EU Air Quality Directive, 2004. Directive 2004/107/EC of the European Parliament and of the Council of 15 December 2004 relating to arsenic, cadmium, mercury, nickel and polycyclic aromatic hydrocarbons in ambient air. Official J., (26.1.2005, L23).

Dvorská A., Lammel G., Klánová J., 2011. Use of diagnostic ratios for studying source apportionment and reactivity of ambient polycyclic aromatic hydrocarbons over Central Europe. Atmos. Environ. 45, 420-427.

Iodice P., Adamo P., Capozzi F., Di Palma A., Senatore A., Spagnuolo V., Giordano S., 2016. Air pollution monitoring using emission inventories combined with the moss bag approach. Sci. Total Environ. 541, 1410-1419.

Greenfield A., Graedel T.E., 2013. The omnivorous diet of modern technology. Resour. Conserv. Recy. 74, 1-7.

INEA, 2011. L’agricoltura nella Campania in cifre, 2010. Roma, p. 154.

Jacques D., Mallants D., Šimůnek J., Van Genuchten Th.M., 2008. Modelling the fate of uranium from inorganic phosphorus fertilizer applications in agriculture. In: De Kok L.J., Schnug E. (Eds.), Loads and Fate of Fertilizer-derived Uranium. Backhuys Publishers, Leiden, The Netherlands, pp. 57-64.

Larsen R.K., Baker J.E., 2003. Source apportionment of polycyclic aromatic hydrocarbons in the urban atmosphere: a comparison of three methods. Environ. Sci. Technol. 37, 1873-1881.

Lelieveld J., Evans J.S., Fnais M., Giannadaki D., Pozzer A., 2015. The contribution of outdoor air pollution sources to premature mortality on a global scale. Nature 525, 367-71. doi:10.1038/nature1537.

Luo Z., Wang Z., Xu B., Sarakiotis I.L., Du Laing G., Yan C., 2014. Measurement and characterization of engineered titanium dioxide nanoparticles in the environment. J. Zhejiang Univ. Sci. A. (Appl. Phys. & Eng.) 15, 593-605.

Mear F., Yot P., Cambon M., Ribes A.M., 2005. Elaboration and characterization of foam glass from cathode ray tubes. Adv. Appl. Ceram. 104, 123-130.

Monaco D., Riccio A., Chianese E., Adamo P., Di Rosa S., Fagnano M., 2015. Chemical characterization and spatial distribution of PAHs and heavy hydrocarbons in rural sites of Campania Region, South Italy. Environ. Sci. Pollut. Res., 14993-15003. doi 10.1007/s11356-015-4733-y.

Nature editorial, 2014. A toxic legacy. Nature 508, 431 (24 April 2014). doi:10.1038/508431a.

Perepezko J.H., 2009. The Hotter the Engine, the Better. Science 326, 1068-1069.

Ravindra K., Sokhia R., Van Griekenb R., 2008. Atmospheric polycyclic aromatic hydrocarbons: Source attribution, emission factors and regulation. Atmos. Environ. 42, 2895–2921.

Sainju U.M., Dris R., Singh B., 2003. Mineral nutrition of tomato. J. Food Agric. Environ. 1, 176-183.

Senior K., Mazza A., 2004. Italian “Triangle of death” linked to waste crisis. Lancet 5, 525-527.

Shimizu T., 1998. Review of Selected Pyrotechnic Publications of Dr. Takeo Shimizu, Part 3: Studies on Fireworks Colored-Flame Compositions. J. Pyro. ISBN 1-889526-11-8.

Singh K.P., Malik A., Kumar R., Saxena P., Sinha S., 2008. Receptor modeling for source apportionment of polycyclic aromatic hydrocarbons in urban atmosphere. Environ. Monit. Assess. 136, 183–196.

Steinnes E., Rühling Å., Lippo H., Makinen A., 1997. Reference materials for large-scale metal deposition surveys. Accred. Qual. Assur. 2, 243-249.

Tobiszewski M., Namieśnik J., 2012. PAH diagnostic ratios for the identification of pollution emission sources. Environ. Pollut. 162, 110-119.

Tretiach M., Pittao E., Crisafulli P., Adamo P., 2011. Influence of exposure sites on trace element enrichment in moss-bags and characterization of particles deposited on the biomonitor surface. Sci. Total Environ. 822, 830-409.

Triassi M., Alfano R., Illario M., Nardone A., Caporale O., Montuori P., 2015. Environmental Pollution from Illegal Waste Disposal and Health Effects: A Review on the “Triangle of Death”. Int. J. Environ. Res. Public Health 12, 1216-1236.

Tyler G., 2004. Rare earth elements in soil and plant systems. Plant Soil 267, 191-206.

Vingiani S., De Nicola F., Purvis W.O., Concha-Graña E., Muniategui-Lorenzo,S., López-Mahía P., Giordano S., Adamo, P., 2015. Active Biomonitoring of Heavy Metals and PAHs with

Mosses and Lichens: a Case Study in the Cities of Naples and London. Water Air Soil Pollut. 226, 1-12.

Vuković G., Urošević M.A., Goryainova Z., Pergal M., Škrivanj S., Samson R., Popović A. 2015. Active moss biomonitoring for extensive screening of urban air pollution: Magnetic and chemical analyses. Sci. Total Environ. 521, 200-210.

Yunker M.B., Macdonald R.W., Vingarzan R., Mitchell R.H., Goyette D., Sylvestre S., 2002. PAHs in the Fraser River basin: a critical appraisal of PAH ratios as indicators of PAH source and composition. Org. Geochem. 33, 489–515.

Sitography

- www.arpacampania.it
- ISTAT BES 2014. Salute. In Rapporto Bes 2014: il benessere equo e sostenibile in Italia. 1, 19-43 http://www.istat.it/it/files/2014/06/01_Salute-Bes2014.pdf
- Legambiente report 2003.
  http://www.internapoli.it/public/legaambiente/REM2003LA10aprile2003.pdf

Supplementary material

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

Table S2. Correlation matrix based on Pearson correlation coefficient of the element accumulated in mosses exposed in agricultural sites. Significant correlations are highlighted in red.

	Al	As	Ca	Cd	Ce	Co	Cr	Cs	Cu	Fe	Hf	Hg	K	La	Mg	Mn	Mo	Na	Nb	Ni	P	Pb	S	Sb	Sc	Se	Sn	Sr	Th	Ti	Tl	U	V	Y	Zn	Zr
Al	1.00	0.05	0.85	0.42	0.93	0.90	0.21	0.94	0.22	0.92	0.24	-0.37	0.60	0.93	0.51	0.86	0.73	0.14	0.89	0.66	0.38	0.73	-0.13	-0.29	-0.28	-0.21	0.17	0.91	0.41	0.88	0.80	0.89	-0.37	0.93	-0.46	0.55
As	0.05	1.00	0.41	0.38	0.07	0.04	0.69	0.03	0.42	0.11	0.14	0.07	-0.12	0.13	0.20	0.10	0.47	-0.02	0.09	-0.09	0.21	-0.03	0.34	0.01	0.14	0.39	0.14	0.27	0.17	-0.06	-0.26	0.12	0.25	0.13	0.16	0.30
Ca	0.85	0.41	1.00	0.32	0.89	0.87	0.42	0.87	0.51	0.93	0.41	-0.41	0.37	0.91	0.47	0.87	0.86	0.06	0.89	0.58	0.27	0.69	-0.11	-0.12	0.06	-0.14	0.43	0.92	0.47	0.85	0.61	0.86	-0.41	0.91	-0.31	0.72
Cd	0.42	0.38	0.32	1.00	0.17	0.31	0.70	0.23	0.40	0.23	-0.03	-0.20	0.34	0.19	0.07	0.11	0.24	-0.04	0.17	0.28	0.29	0.13	0.08	-0.08	-0.40	0.30	0.03	0.26	0.57	0.09	0.02	0.28	0.25	0.19	-0.50	0.13
Ce	0.93	0.07	0.89	0.17	1.00	0.94	0.14	0.99	0.16	0.96	0.26	-0.40	0.59	0.99	0.57	0.95	0.77	0.21	0.98	0.62	0.36	0.73	-0.09	-0.34	-0.16	-0.17	0.16	0.95	0.29	0.96	0.81	0.91	-0.41	1.00	-0.25	0.60
Co	0.90	0.04	0.87	0.31	0.94	1.00	0.29	0.92	0.28	0.97	0.31	-0.35	0.67	0.91	0.57	0.94	0.70	0.27	0.88	0.72	0.46	0.72	-0.02	-0.17	-0.01	-0.22	0.29	0.92	0.41	0.93	0.80	0.82	-0.39	0.93	-0.26	0.55
Cr	0.21	0.69	0.42	0.70	0.14	0.29	1.00	0.15	0.55	0.20	-0.16	-0.22	0.13	0.17	0.16	0.16	0.19	0.01	0.12	-0.07	0.21	0.09	0.27	0.10	0.03	0.27	0.11	0.31	0.36	0.09	-0.14	0.14	0.26	0.18	-0.17	0.08
Cs	0.94	0.03	0.87	0.23	0.99	0.92	0.15	1.00	0.15	0.93	0.19	-0.47	0.59	0.99	0.52	0.92	0.72	0.17	0.98	0.59	0.32	0.73	-0.14	-0.38	-0.26	-0.16	0.10	0.93	0.32	0.96	0.80	0.93	-0.39	0.99	-0.34	0.57
Cu	0.22	0.42	0.51	0.40	0.16	0.28	0.55	0.15	1.00	0.31	0.55	-0.47	-0.32	0.21	-0.36	0.16	0.40	-0.49	0.24	0.22	-0.34	0.18	-0.43	0.35	0.41	-0.03	0.82	0.17	0.76	0.27	-0.21	0.37	-0.33	0.20	-0.51	0.63
Fe	0.92	0.11	0.93	0.23	0.96	0.97	0.20	0.93	0.31	1.00	0.45	-0.34	0.57	0.95	0.56	0.96	0.82	0.21	0.92	0.75	0.41	0.75	-0.08	-0.18	0.03	-0.25	0.38	0.95	0.42	0.94	0.80	0.87	-0.47	0.96	-0.25	0.67
Hf	0.24	0.14	0.41	-0.03	0.26	0.31	-0.16	0.19	0.55	0.45	1.00	-0.05	-0.12	0.28	-0.11	0.36	0.65	-0.22	0.31	0.65	0.00	0.28	-0.31	0.14	0.54	-0.21	0.84	0.22	0.57	0.31	0.14	0.37	-0.55	0.28	-0.11	0.79
Hg	-0.37	0.07	-0.41	-0.20	-0.40	-0.35	-0.22	-0.47	-0.47	-0.34	-0.05	1.00	0.22	-0.44	0.41	-0.32	-0.26	0.67	-0.53	0.03	0.54	-0.55	0.77	0.40	0.14	0.22	-0.26	-0.24	-0.65	-0.49	-0.13	-0.56	0.57	-0.41	0.78	-0.50
K	0.60	-0.12	0.37	0.34	0.59	0.67	0.13	0.59	-0.32	0.57	-0.12	0.22	1.00	0.52	0.81	0.56	0.25	0.79	0.45	0.63	0.82	0.23	0.52	-0.14	-0.39	0.15	-0.32	0.63	-0.12	0.50	0.68	0.38	0.26	0.56	0.18	-0.08
La	0.93	0.13	0.91	0.19	0.99	0.91	0.17	0.99	0.21	0.95	0.28	-0.44	0.52	1.00	0.52	0.94	0.80	0.13	0.98	0.58	0.32	0.74	-0.13	-0.38	-0.16	-0.16	0.18	0.95	0.34	0.95	0.77	0.93	-0.43	1.00	-0.29	0.66
Mg	0.51	0.20	0.47	0.07	0.57	0.57	0.16	0.52	-0.36	0.56	-0.11	0.41	0.81	0.52	1.00	0.61	0.37	0.84	0.41	0.43	0.87	0.29	0.70	-0.07	-0.10	0.00	-0.27	0.72	-0.36	0.42	0.69	0.26	0.19	0.56	0.46	-0.09
Mn	0.86	0.10	0.87	0.11	0.95	0.94	0.16	0.92	0.16	0.96	0.36	-0.32	0.56	0.94	0.61	1.00	0.80	0.22	0.90	0.63	0.47	0.81	-0.01	-0.35	0.07	-0.33	0.26	0.94	0.33	0.90	0.84	0.80	-0.51	0.95	-0.14	0.62
Mo	0.73	0.47	0.86	0.24	0.77	0.70	0.19	0.72	0.40	0.82	0.65	-0.26	0.25	0.80	0.37	0.80	1.00	-0.05	0.79	0.61	0.33	0.66	-0.10	-0.34	0.11	-0.12	0.45	0.79	0.52	0.67	0.52	0.78	-0.46	0.80	-0.19	0.86
Na	0.14	-0.02	0.06	-0.04	0.21	0.27	0.01	0.17	-0.49	0.21	-0.22	0.67	0.79	0.13	0.84	0.22	-0.05	1.00	0.05	0.35	0.78	-0.21	0.82	0.23	-0.13	0.28	-0.40	0.33	-0.60	0.14	0.37	-0.06	0.54	0.19	0.67	-0.40
Nb	0.89	0.09	0.89	0.17	0.98	0.88	0.12	0.98	0.24	0.92	0.31	-0.53	0.45	0.98	0.41	0.90	0.79	0.05	1.00	0.58	0.19	0.72	-0.25	-0.39	-0.20	-0.12	0.20	0.89	0.38	0.95	0.72	0.97	-0.46	0.98	-0.35	0.70
Ni	0.66	-0.09	0.58	0.28	0.62	0.72	-0.07	0.59	0.22	0.75	0.65	0.03	0.63	0.58	0.43	0.63	0.61	0.35	0.58	1.00	0.47	0.37	0.00	0.08	0.01	-0.06	0.42	0.59	0.40	0.63	0.61	0.60	-0.25	0.61	-0.12	0.51
P	0.38	0.21	0.27	0.29	0.36	0.46	0.21	0.32	-0.34	0.41	0.00	0.54	0.82	0.32	0.87	0.47	0.33	0.78	0.19	0.47	1.00	0.17	0.80	-0.13	-0.09	0.11	-0.26	0.53	-0.19	0.19	0.52	0.09	0.31	0.35	0.46	-0.10
Pb	0.73	-0.03	0.69	0.13	0.73	0.72	0.09	0.73	0.18	0.75	0.28	-0.55	0.23	0.74	0.29	0.81	0.66	-0.21	0.72	0.37	0.17	1.00	-0.35	-0.47	0.08	-0.69	0.31	0.72	0.52	0.67	0.79	0.61	-0.78	0.73	-0.48	0.53
S	-0.13	0.34	-0.11	0.08	-0.09	-0.02	0.27	-0.14	-0.43	-0.08	-0.31	0.77	0.52	-0.13	0.70	-0.01	-0.10	0.82	-0.25	0.00	0.80	-0.35	1.00	0.13	-0.01	0.40	-0.46	0.12	-0.58	-0.23	0.02	-0.34	0.70	-0.09	0.79	-0.46
Sb	-0.29	0.01	-0.12	-0.08	-0.34	-0.17	0.10	-0.38	0.35	-0.18	0.14	0.40	-0.14	-0.38	-0.07	-0.35	-0.34	0.23	-0.39	0.08	-0.13	-0.47	0.13	1.00	0.46	0.03	0.42	-0.25	-0.18	-0.20	-0.30	-0.34	0.21	-0.34	0.18	-0.24
Sc	-0.28	0.14	0.06	-0.40	-0.16	-0.01	0.03	-0.26	0.41	0.03	0.54	0.14	-0.39	-0.16	-0.10	0.07	0.11	-0.13	-0.20	0.01	-0.09	0.08	-0.01	0.46	1.00	-0.45	0.71	-0.08	0.13	-0.08	-0.11	-0.26	-0.42	-0.14	0.27	0.24
Se	-0.21	0.39	-0.14	0.30	-0.17	-0.22	0.27	-0.16	-0.03	-0.25	-0.21	0.22	0.15	-0.16	0.00	-0.33	-0.12	0.28	-0.12	-0.06	0.11	-0.69	0.40	0.03	-0.45	1.00	-0.40	-0.17	-0.20	-0.20	-0.51	-0.01	0.82	-0.15	0.30	-0.12
Sn	0.17	0.14	0.43	0.03	0.16	0.29	0.11	0.10	0.82	0.38	0.84	-0.26	-0.32	0.18	-0.27	0.26	0.45	-0.40	0.20	0.42	-0.26	0.31	-0.46	0.42	0.71	-0.40	1.00	0.15	0.66	0.28	0.03	0.26	-0.63	0.18	-0.33	0.67
Sr	0.91	0.27	0.92	0.26	0.95	0.92	0.31	0.93	0.17	0.95	0.22	-0.24	0.63	0.95	0.72	0.94	0.79	0.33	0.89	0.59	0.53	0.72	0.12	-0.25	-0.08	-0.17	0.15	1.00	0.24	0.87	0.81	0.80	-0.30	0.96	-0.14	0.51
Th	0.41	0.17	0.47	0.57	0.29	0.41	0.36	0.32	0.76	0.42	0.57	-0.65	-0.12	0.34	-0.36	0.33	0.52	-0.60	0.38	0.40	-0.19	0.52	-0.58	-0.18	0.13	-0.20	0.66	0.24	1.00	0.33	0.08	0.49	-0.54	0.31	-0.73	0.71
Ti	0.88	-0.06	0.85	0.09	0.96	0.93	0.09	0.96	0.27	0.94	0.31	-0.49	0.50	0.95	0.42	0.90	0.67	0.14	0.95	0.63	0.19	0.67	-0.23	-0.20	-0.08	-0.20	0.28	0.87	0.33	1.00	0.74	0.92	-0.47	0.96	-0.33	0.63
Tl	0.80	-0.26	0.61	0.02	0.81	0.80	-0.14	0.80	-0.21	0.80	0.14	-0.13	0.68	0.77	0.69	0.84	0.52	0.37	0.72	0.61	0.52	0.79	0.02	-0.30	-0.11	-0.51	0.03	0.81	0.08	0.74	1.00	0.58	-0.47	0.78	-0.13	0.25
U	0.89	0.12	0.86	0.28	0.91	0.82	0.14	0.93	0.37	0.87	0.37	-0.56	0.38	0.93	0.26	0.80	0.78	-0.06	0.97	0.60	0.09	0.61	-0.34	-0.34	-0.26	-0.01	0.26	0.80	0.49	0.92	0.58	1.00	-0.40	0.92	-0.47	0.77
V	-0.37	0.25	-0.41	0.25	-0.41	-0.39	0.26	-0.39	-0.33	-0.47	-0.55	0.57	0.26	-0.43	0.19	-0.51	-0.46	0.54	-0.46	-0.25	0.31	-0.78	0.70	0.21	-0.42	0.82	-0.63	-0.30	-0.54	-0.47	-0.47	-0.40	1.00	-0.40	0.49	-0.61
Y	0.93	0.13	0.91	0.19	1.00	0.93	0.18	0.99	0.20	0.96	0.28	-0.41	0.56	1.00	0.56	0.95	0.80	0.19	0.98	0.61	0.35	0.73	-0.09	-0.34	-0.14	-0.15	0.18	0.96	0.31	0.96	0.78	0.92	-0.40	1.00	-0.26	0.63
Zn	-0.46	0.16	-0.31	-0.50	-0.25	-0.26	-0.17	-0.34	-0.51	-0.25	-0.11	0.78	0.18	-0.29	0.46	-0.14	-0.19	0.67	-0.35	-0.12	0.46	-0.48	0.79	0.18	0.27	0.30	-0.33	-0.14	-0.73	-0.33	-0.13	-0.47	0.49	-0.26	1.00	-0.39
Zr	0.55	0.30	0.72	0.13	0.60	0.55	0.08	0.57	0.63	0.67	0.79	-0.50	-0.08	0.66	-0.09	0.62	0.86	-0.40	0.70	0.51	-0.10	0.53	-0.46	-0.24	0.24	-0.12	0.67	0.51	0.71	0.63	0.25	0.77	-0.61	0.63	-0.39	1.00

General Conclusions

The biomonitoring with “moss-bags” transplants is a methodology not yet proposed by the EU legislation and hence rarely applied by territorial authorities involved in air pollution control, mainly because there is a lack of standardized procedures, the atmospheric content of pollutants cannot be inferred from the amount of deposited matter intercepted during the exposure period (mostly for the lack of instrumental data to be used in the inter-calibration analysis) and threshold values indicating the degree of exposure risk are missing. On the other side, this methodology can give precious information to rebuilt fall-out patterns from diffuse or single emitting sources, to evaluate temporal trends, to identify hot-spots of pollution when the aim is to plan the location of instrumental recording gauges.

The main aim of this PhD thesis was actually to standardize the “moss-bags” methodology, and two field experiments were performed, making comparisons that were never or insufficiently tested before, with the aim to demonstrate the effectiveness of this technique.

In the first work (**Chapter 1**), the potentialities of a devitalized clone of *S. palustre* as an excellent biomaterial for the active monitoring of persistent air pollutants were shown. The comparison between the elemental concentration of *S. palustre* collected in background, pristine sites and those of differently treated clones of the same specie cultivated in highly standardized conditions clearly showed that the latter have lower and homogenous element concentrations. Moreover, the laboratory production can solve the problems linked to the uncontrolled harvesting of material in the wilderness; in addition, it enables untrained people, involved in the application of the methodology, to work with the warranty of having a marketed homogeneous material. This solution is also eco-friendly, since the clone needs only the oven-devitalization (necessary to preserve the biomaterial, it ensures its easy handling and storing, and eliminates the variability due to possible differences in moss growth rate during exposure). The EDTA washing, although essential for native species, is unnecessary for the clone whose elemental content is very low. These characteristics indicate *S palustre* as particularly suitable to monitor atmospheric depositions also in low polluted environments and for short exposure periods. Our results were also confirmed by a field test, in which the *S. palustre* clone showed a significantly higher metal uptake compared to wild *Pseudoscleropodium purum.* Therefore, the use of the *S. palustre* clone, with its low and stable elemental signature, should be taken into account in air pollution biomonitoring, being a highly performing material.

In the second work (**Chapter 2**), for the first time a systematic experimental design, testing separately the various variables affecting moss uptake in bag, has been performed in three countries differing for climate and meteorology and, in each country, choosing sites belonging to diverse land use classes, in order to single out the best exposure solutions. The results evidenced that the shape of the bags and the mesh size do not affect the accumulation capacity of the exposed moss, whereas the density of the moss inside the bags directly influences the uptake capacity. Moreover, it has been evidenced that three weeks of exposure were inadequate to get a detectable accumulation, while only a small difference were observed between the 6- and 12-week periods of exposure. Finally, the height experiment did not show significant differences among the three tested standard heights (4, 7, 10 meters above the ground). According to the reported outcomes, we consider preferable to use a Mossphere (being not home-made, reusable and with a fixed shape) with a 2 mm mesh and a weight/surface ratio ranging between 5 up to 15 mg cm^{-2} exposed for a period not lower than 6 weeks (for an increased temporal resolution) at 4 meters above the ground (merely for practical reasons).

In **Chapter 3**, it was demonstrated that the combined use of two methods for evaluating air pollution, the inventory of emission sources and biomonitoring by moss transplants, could be a useful tool to study air quality and set up possible remediation plans. When the emission inventory is based on a reliable and constant-over-time single pollution source, the two data sets showed coincident outputs, whereas less overlapping outputs were obtained for those contaminants showing multiple and scattered sources, variable in time. Therefore, the joint use of the two approaches could be particularly useful in areas of alleged pollution to support the monitoring of attainment of limit values established by the current legislation. The comparison between the two data sets (i.e. emission inventory and biomonitoring) allowed to reveal purported pollution point sources and the simultaneous detection of contaminants not monitored by automatic devices or eluding the emission inventories.

In the fourth experiment (**Chapter 4**), through the exposure scheme adopted, it was proved that *Hypnum cupressiforme* “moss-bags” are able to discriminate different pollution levels in agricultural and urban scenarios and green and street sub-scenarios in a landscape characterized by a jeopardized structure, where these sites are strictly mixed together. In particular, we found that the agricultural sites can be more impacted, in terms of moss uptake, than urban ones. At a regional scale the impact of agricultural practices on air quality was fully circumstantiated; this result seems to be in line with a global trend, since agriculture has recently been reported as the leading source of particulate matter (specifically $PM_{2.5}$) in Europe. Such a detailed information, down to a very small land scale, would never be possible by using monitoring stations, also considering the large set of pollutants here analysed (i.e. 39 chemical elements and 20 PAHs).

All together, the results seem to give fully support to the adoption of the moss bag technique at EU level in order to improve data quality in the field of air pollution monitoring.

Appendix

Conferences and outreach contributions:

Aboal J., Adamo P., Bargagli R., Beike A.K., Bowkett M., Capozzi F., Carballeira C. B., Ceolin A., Concha-Graña E., Crespo Pardo D., Decker E. L., Di Palma A., Fernandez J. A., Fernandez V., Giordano S., Gonzalez A. G., Iglesias-Samitier S., Lopez Mahia P., MUniategui S., Pokrovsky O. S., Reski R., Rey-Asensio A. I., Spagnuolo V., Tretiach M., 2015. “A novel biotechnological tool to monitor air quality based on a devitalized moss clone: the MOSSclone output”. XXV° Congresso EEF/SItE - “ECOLOGY AT THE INTERFACE”, 21-25, settembre, Roma.

Adamo P., Giordano S., Capozzi F., De Nicola F., Di Palma A., Iodice P., Senatore A., Spagnuolo V., 2015. “Biomonitoring Research of Heavy Metals and PAHs in the Air of Agricultural Landscape of Italy’s Campania Region”. Book of abstract of the 7th International Workshop on Biomonitoring of Atmospheric Pollution (BIOMAP), June 14-19, Lisbon, Portugal

Capozzi F., Cecconi E., Adamo P., Bargagli R., Benesperi R., Bidussi M., Candotto Carniel F., Craighero T., Cristofolini F., Giordano S., Panepinto F., Puntillo D., Ravera S., Spagnuolo V., Tretiach M., 2015. “Contenuto elementare nei talli del lichene epifita Pseudevernia furfuracea (L.) Zopf. raccolti in aree remote d’Italia” Notiziario della Società Lichenologica Italiana, Atti del 28° Congresso, 9-11 novembre, Lanciano (CH).

Giordano S., Aboal J., Adamo P., Bargagli R., Beike A.K., Bowkett M., Capozzi F., Carballeira C. B., Ceolin A., Concha-Graña E., Crespo Pardo D., Decker E. L., Di Palma A., Fernandez J. A., Fernandez V., Gonzalez A. G., Iglesias-Samitier S., Lopez Mahia P., MUniategui S., Pokrovsky O. S., Reski R., Rey-Asensio A. I., Spagnuolo V., Tretiach M., 2015. “Creating and Testing a Novel Biotechnological Tool to Monitor Air Quality Based on a Devitalized Moss Clone”. Book of abstract of the 7th International Workshop on Biomonitoring of Atmospheric Pollution (BIOMAP), June 14-19, Lisbon, Portugal

Panepinto F., Capozzi F., Tretiach M., 2015. “Fattori limitanti la ricolonizzazione lichenica in ambiente urbano”. Notiziario della Società Lichenologica Italiana, Atti del 28° Congresso, 9-11 novembre, Lanciano (CH).

Capozzi F., Adamo P., Bargagli R., Di Palma A., Giordano S., Spagnuolo V., Terracciano S., Tretiach M., 2014. ”Produzione e test di un muschio clonato e devitalizzato come biomonitor attivo di elementi in traccia: l’esperienza del progetto FP7 Mossclone”. Notiziario della Società Lichenologica Italiana, Atti del 27° Congresso, 15-17 ottobre, Montecatini Terme (PT).

Fortuna L., Candotto Carniel F., Capozzi F., Panepinto F., Tretiach M., 2014. “Biomonitoraggio in territori eterogenei: un’occasione per confrontare la capacità di bioaccumulo di due specie allopatriche”. Notiziario della Società Lichenologica Italiana, Atti del 27° Congresso, 15-17 ottobre, Montecatini Terme (PT).

Panepinto F., Capozzi F., Craighero T., Tretiach M., 2014. “Studio sui fattori limitanti la ricolonizzazione lichenica in ambiente urbano: il ruolo dell’umidità atmosferica”. Notiziario della Società Lichenologica Italiana, Atti del 27° Congresso, 15-17 ottobre, Montecatini Terme (PT).

Di Palma A., Adamo P., Bargagli R., Capozzi F., Iavazzo P., Spagnuolo V., Terracciano S., Tretiach M., Giordano S., 2013. “MOSSCLONE: A EU-FP7 project to standardize a devitalized moss clone as passive contaminant sensor”. XXXI Convegno Nazionale della Società Italiana di Chimica Agraria.

Fortuna L., Candotto Carniel F., Capozzi F., Panepinto F., Tretiach M., 2013. “Proposta metodologica per una indagine speditiva a supporto di piani di biomonitoraggio mediante licheni quali bioaccumulatori di metalli”. Notiziario della Società Lichenologica Italiana, Atti del 26° Congresso, 2-4 ottobre, Piacenza (PC).

Tretiach M., Adamo P., Bargagli R., Capozzi F., Di Palma A., Iavazzo P., Spagnuolo V., Terracciano S., Giordano S., 2013. “Aspetti metodologici del monitoraggio di inquinanti aerodiffusi persistenti mediante “moss-bags” di nuova generazione”. Notiziario della Società Lichenologica Italiana, Atti del 26° Congresso, 2-4 ottobre, Piacenza (PC).

Acknowledgements

Prima di tutto voglio ringraziare i miei supervisori, Mauro Tretiach e Simonetta Giordano: siete esempio di impegno e dedizione al lavoro e per questo mi considero molto fortunato ad aver lavorato con voi!

Molte persone meritano la mia gratitudine…spero di non dimenticarne nessuna…..☺…

Grazie “Geobotanici” con voi ho iniziato e ho condiviso gran parte di questo “tortuoso” percorso: ad Alice, che mi/ci ha nutrito con i suoi taralli…Pugliesi!?..Veneti!?...forse da “Reggio Catania”!?; a Danijela, che ha sempre risposto alle mie richieste d’aiuto; ad Elena, un appoggio prezioso in ogni circostanza; a Fabio e Stefano, i primi ad accogliermi in quel freddo gennaio del 2013; a Francesco, con il quale sin dall’inizio abbiamo messo in atto il teatrino del: [F:] “*offro io il caffè*!”…[Io:] “*no no faccio io*!”…[F:] “*no no, sono il più anziano faccio io*!”…[Io:] “*allora farai sempre tu*!!?”…; a Lorenzo, collega in più attività di campo…abbiamo rischiato anche di rimanere “impantanati”!; a Massimo, compagno di “ciaspola” (caratterizzato da una falcata doppia rispetto alla mia, del tipo che per fare 10 m lui 3 passi io 10!!)…che mi ha dato anche preziosi consigli sulla redazione della presente tesi; a Teresa, che, nonostante si fosse liberato un posto (quello di Fabio partito per Innsbruck) nell’anno in cui ha iniziato il suo dottorato, ha deciso di stanziarsi sulla mia scrivania!!!!… “tnx b****!!!”...;-).

Grazie ad Elva con cui ho condiviso stancanti giornate nella logorante attività delle “polverizzazioni” per il progetto “Furfy”…

Grazie alle persone che ho conosciuto nel corso dei progetti Life e Mossclone dalle quali ho appreso veramente tanto. In particolare: ad Anna, con cui ho lavorato ad entrambi i progetti e che mi ha insegnato ad essere attento ai particolari; a Pietro e al caro Stefano, i primi ricercatori con cui ho avuto un esperienza in “campo”; alla Prof. Paola Adamo e alla Prof. Valeria Spagnuolo che, nonostante i mille impegni, hanno sempre trovato tempo da dedicarmi; i colleghi spagnoli Angel, Jesus e Zulema, con i quali ho avuto il piacere di lavorare all’elaborazione dei dati ottenuti dal “Mossclone.

Ringrazio infine mia moglie Sabina, dal momento che se non fosse riuscita a convincermi a partecipare al concorso per questo dottorato, adesso non starei scrivendo questi ringraziamenti!

Fiore