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

F. Capozzi [a, h], S. Giordano [a, *], J.R. Aboal [b], P. Adamo [c], R. Bargagli [d], T. Boquete [b], A. Di Palma [c], C. Real [b], R. Reski [e, f], V. Spagnuolo [a], K. Steinbauer [g], M. Tretiach [h], Z. Varela [i], H. Zechmeister [j], J.A. Fernández [b]

[a] *Dipartimento di Biologia, Università di Napoli Federico II, Campus Monte S. Angelo, Via Cinthia 4, 80126 Napoli, Italy*
[b] *Department of Cellular Biology and Ecology, Faculty of Biology, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain*
[c] *Dipartimento di Agraria, Università di Napoli Federico II, Via Università, 100, 80055 Portici (NA), Italy*
[d] *Dipartimento di Scienze Fisiche, della Terra e dell'Ambiente, Università di Siena, Via P.A. Mattioli 4, 53100 Siena, Italy*
[e] *Plant Biotechnology, Faculty of Biology, University of Freiburg, Schaenzlestr. 1, 79104 Freiburg, Germany*
[f] *BIOSS – Centre for Biological Signalling Studies, 79104 Freiburg, Germany*
[g] *GLORIA-Coordination, Institute for Interdisciplinary Mountain Research, Austrian Academy of Sciences & Center for Global Change and Sustainability, University of Natural Resources and Life Sciences Vienna, Austria*
[h] *Dipartimento di Scienze della Vita, Università di Trieste, Via L. Giorgieri 10, 34127 Trieste, Italy*
[i] *BIOVIA Consultor Ambiental, Edificio Emprendia, Campus Vida, 15782 Santiago de Compostela, Spain*
[j] *Dept. Botany and Biodiversity Research, University of Vienna, Rennweg 14, 1030 Vienna, Austria*

ARTICLE INFO

Keywords:
Active biomonitoring
Air pollution
Moss uptake
Trace elements
Mossphere
Pseudoscleropodium purum

ABSTRACT

To develop an internationally standardized protocol for the moss bag technique application, 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 carried out 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 uptake 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 uptake 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. Even though 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). Even if some metals in (wet, dry and occult)

☆ This paper has been recommended for acceptance by Hageman Kimberly Jill.
* Corresponding author.
E-mail address: giordano@unina.it (S. Giordano).

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 air quality 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 pattern of 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 (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. To develop an internationally standardized protocol for the moss bag technique it seemed necessary to test the variability in each of the methodological steps in a range of climatic conditions and land use classes. To this end an experimental design was, for the first time, applied in urban, industrial, agricultural and background areas of three European countries with oceanic, Mediterranean and continental climate.

2. Material and methods

2.1. Preparation of moss transplants

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

All moss samples were carefully separated from litter and other exogenous materials and air-dried in laboratory. For the experiments, only 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 h each at 50 °C, 80 °C and 100 °C (modified from: Adamo et al., 2007; Giordano et al., 2009). 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; Tables S1, S2, S3). 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 and 1200 mm (http://www.sito.regione.campania.it/). Austria is not bordering the sea and presenting a temperate/continental climate in the investigated area (annual rainfall 550–900 mm; annual average temperature, 11.3 °C and spring average, 6.5 °C) (http://www.zamg.ac.at/).

In each country seven exposure sites (ESs) affected by different level 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 °C, 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} -, Fig. 2.

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

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

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

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

2.2.2. Mesh size

Mosspheres with different mesh size (1 mm, 2 mm, and 4 mm)

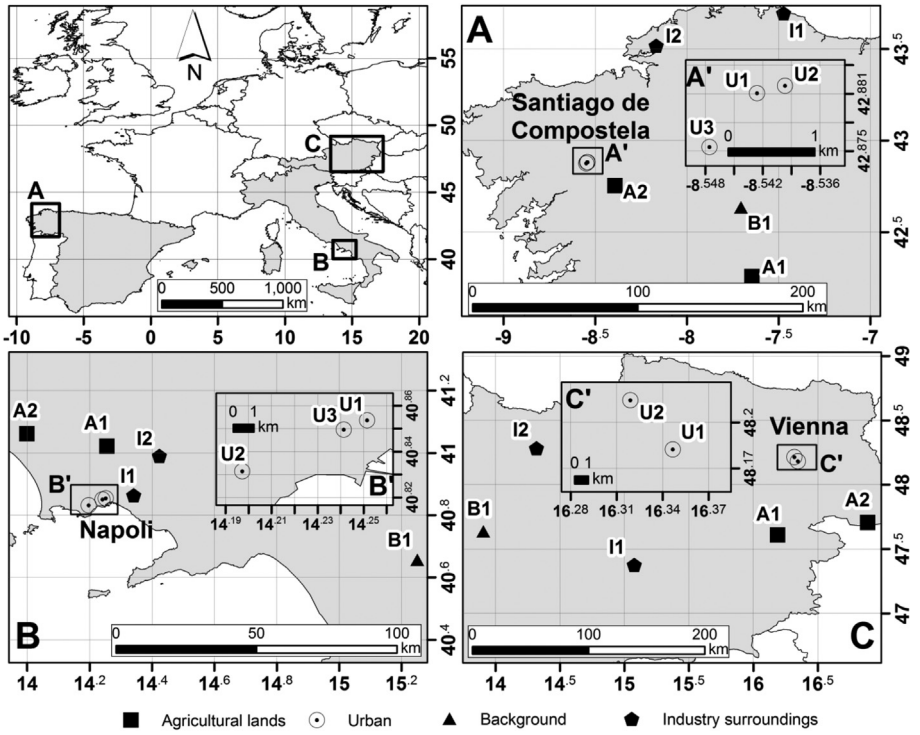

Fig. 1. Maps showing the seven stations distributed in industrial, urban, agricultural and background scenarios in Spain (A), Italy (B) and Austria (C).

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

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 × 3 countries × 7 ESs × 3 replicates).

2.2.3. Ratio between moss weight and bag surface area

In order to investigate the effect of weight, Mossphere 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 Mossphere 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 wt/surface ratios × 3 countries × 7 ESs × 3 replicates).

2.2.4. Duration of exposure

For this assay, Mossphere 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 × 7 EEs × 4 periods × 3 replicates), 2 subsequent exposure periods of 6 weeks (n = 126; 3 countries × 7 EEs × 2 periods × 3 replicates) and 1 exposure period of 12 weeks (n = 63; 3 countries × 7 EEs × 3 replicates).

2.2.5. Height of exposure

For this assay, Mossphere 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 × 5 ESs × 3 heights × 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 mosses were dried at 40 °C until constant weight. The moss tissue was separately

homogenized from each bag (Total No. of bag processed: 1143), in heavy metal-free mills (Retsch ZM 200 and Retsch PM100). Moss sub-samples of 0.5 g were then digested in 1 mL H_2O_2 (30%, Sigma Aldrich) 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). Digestion solutions without mosses (one solution every 10 samples) were used as blanks and analysed in parallel. 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 (M3 *Pleurozium schreberi*; Steinnes et al., 1997) was analysed in parallel, 1 every 10 samples. Contamination during processing was controlled for by the use of analytical blanks (1 every 10 samples analysed).

Recovery of elements from the reference materials 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 (calculated as – [(|X–V|)/V]*100 -, where X is the mean concentration detected by ICP-MS and V the certified value of M3) 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 below the detection limit. This outcome was not due to the analytical method applied since we obtained good percentages of recovery (section 2.3). Probably, in the chosen sites, some elements were present at low concentrations and the exposure period (section 3.5) was too short. Moreover, the three countries were characterized by different pollution levels: Italy > Austria > Spain (according to the EEA reports "Air quality in Europe" 2012–2013), consequently only those elements complying with the criteria explained above were considered (section 2.4).

3.2. Shape of the bags

According to the data analysis criteria described in the section 2.4, 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. S_{15} mossphere

Fig. 3 shows the comparisons for this assay, separately for each country, associated to the statistical significance. 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 uptake 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 enrichment were observed for the remaining elements. A general trend is clear for the enrichment 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 uptake performance more than the shape *per se*. In terms of data spread, no clear differences were evidenced by the MAD/median ratio (Table 1).

Gailey and Lloyd (1986d) reported a better accumulation by the moss *Hypnum* sp. exposed in rounded bags with respect to the flat bags; although this is not evident in our results, we support the use of (sub-)spherical bags since they allow a uniform collection efficiency from all space directions. The flat bags in which two dimensions are prevalent on the third one, besides being a handmade product, have the problem that the moss uptake can depend on the exposure orientation especially in our set-up because we fixed the bag with two cable ties enabling only one degree of freedom (i.e. rotation around its holder). If the pollutants come from a specific direction, the orientation of a flat bag may largely affect the concentrations found in the moss. Besides, the upper and the lower sides of the flat bags are consistently smaller than the other two, reducing the collection of the particulate matter falling from the top or re-suspended from the soil. 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. S_{30} 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 uptake for Ni and Zn, while Al and Sr where higher in the moss contained in S_{30}. In Spain, the enrichment of Al, Cu, Ni and Zn was higher in the rounded bags, while no differences were found for the remaining elements (Fig. 3). There is a clear general trend only for Zn, for which the rounded bag always allowed the highest uptake. No clear differences in terms of data spread were evidenced by the MAD/median ratio (Table 1). This comparison did not yield a clear result; the two options behaved quite similarly, likely for the same density of the moss material inside the bag (see paragraph 3.4). Probably the higher moss density, joint to the

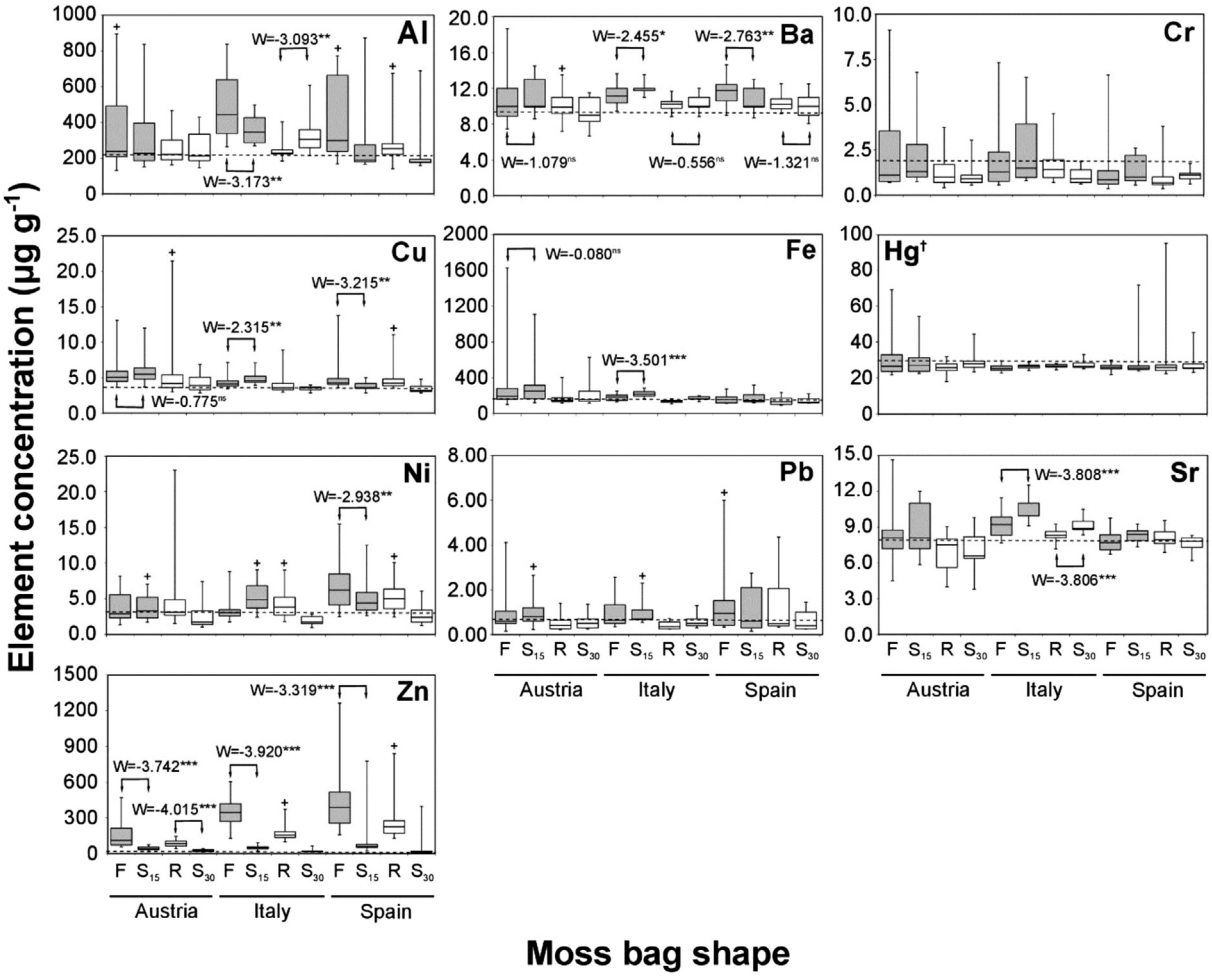

Fig. 3. Box-plots of element concentrations (µg g^{-1}) in the moss exposed in bags of different shape in Austria, Italy and Spain (flat = F *vs* S_{15} Mossphere, grey; rounded = R *vs* S_{30} Mossphere, white). The dashed line represents the LOQ_T. BOX: inside band = median; extremities = 1st and 3rd quartiles; whiskers = MIN and MAX. W is the value of Wilcoxon test. "+" indicates the only option(s) fulfilling our criterion; *p < 0.05, **p < 0.01, ***p < 0.001.

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

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. 4). 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 uptake 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 uptake 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 uptake (section 3.4). No clear differences in data spread were found for different mesh sizes (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 enrichment and this seemed true independently of the mesh size interval employed. According to different studies, the choice of an

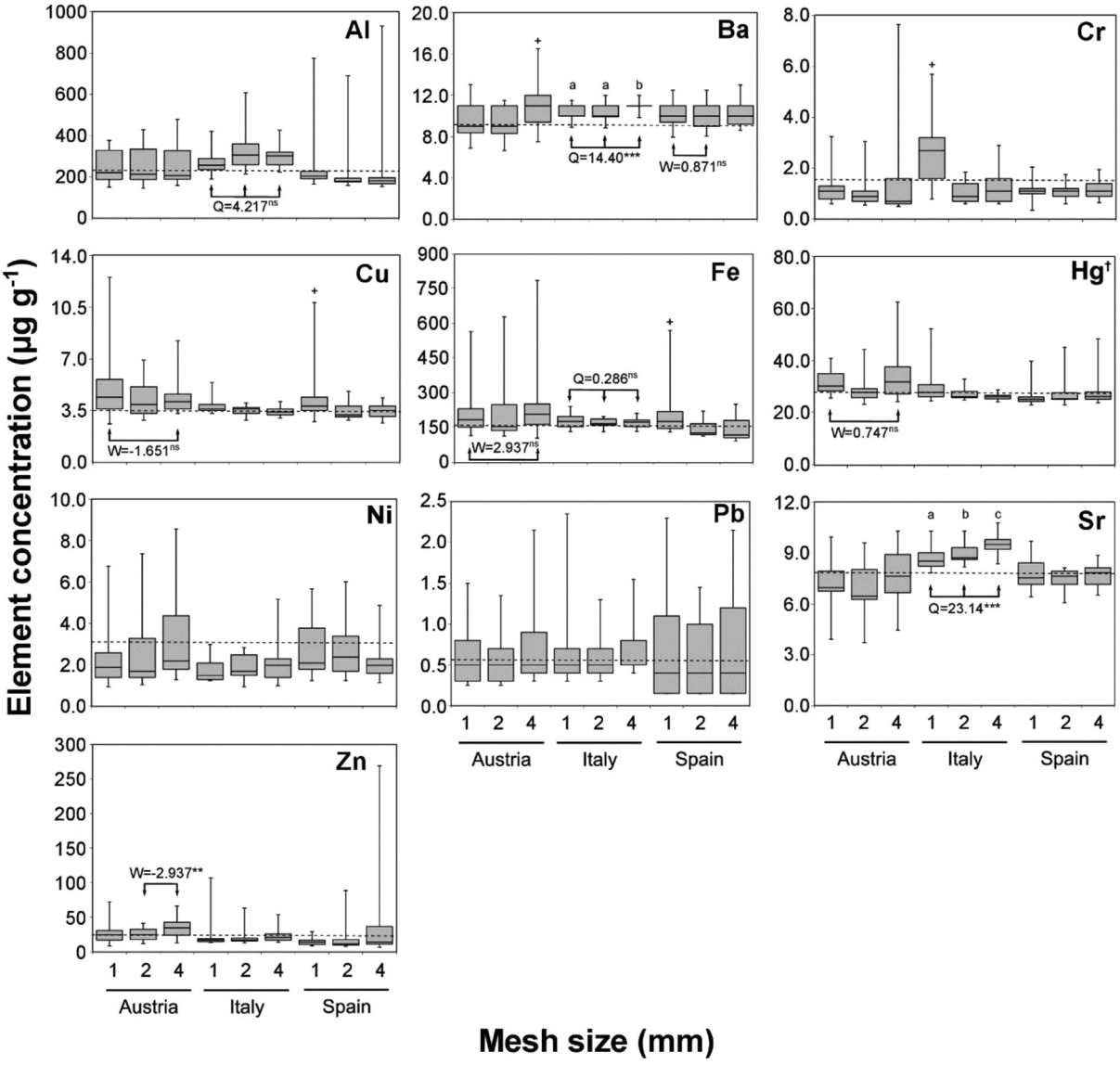

Fig. 4. Box-plots of element concentrations (µg g^{-1}) in the moss exposed in Mosspheres with different mesh sizes (1, 2, 4 mm) in Austria, Italy and Spain. The grey dashed line represents the LOQ_T. BOX: inside band = median; extremities = 1st and 3rd quartiles; whiskers = MIN and MAX. W, Q are the values of Wilcoxon and Friedman ANOVA tests. "+" indicates the only option(s) fulfilling our criterion; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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

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 uptake of elements in moss in a significant way; the selection of the proper mesh size must thus take into account only the loss of material during the exposure.

3.4. Ratio between moss weight and bag surface area

It was not possible to carry out the statistical analysis for Cr, as most of the data did not satisfy the 60% criterion. For all the remaining elements, the final concentrations increased in the Mossphere filled with the smallest amount of moss. The W15 was the only option fulfilling the LOQ_T for most of the elements as the case of: Ba, Fe, Ni and Pb in Austria; Pb and Ni in Italy; Cu, Ni and Sr in Spain. In all the other circumstances, when W30 and W45 also fulfilled the criterion, W15 significantly differed from the other two setups giving always the "largest signal" (Fig. 5). In replicability terms no clear trend was evidenced by the ratio between median absolute deviation (MAD) and median (Table 3). Our results reflect

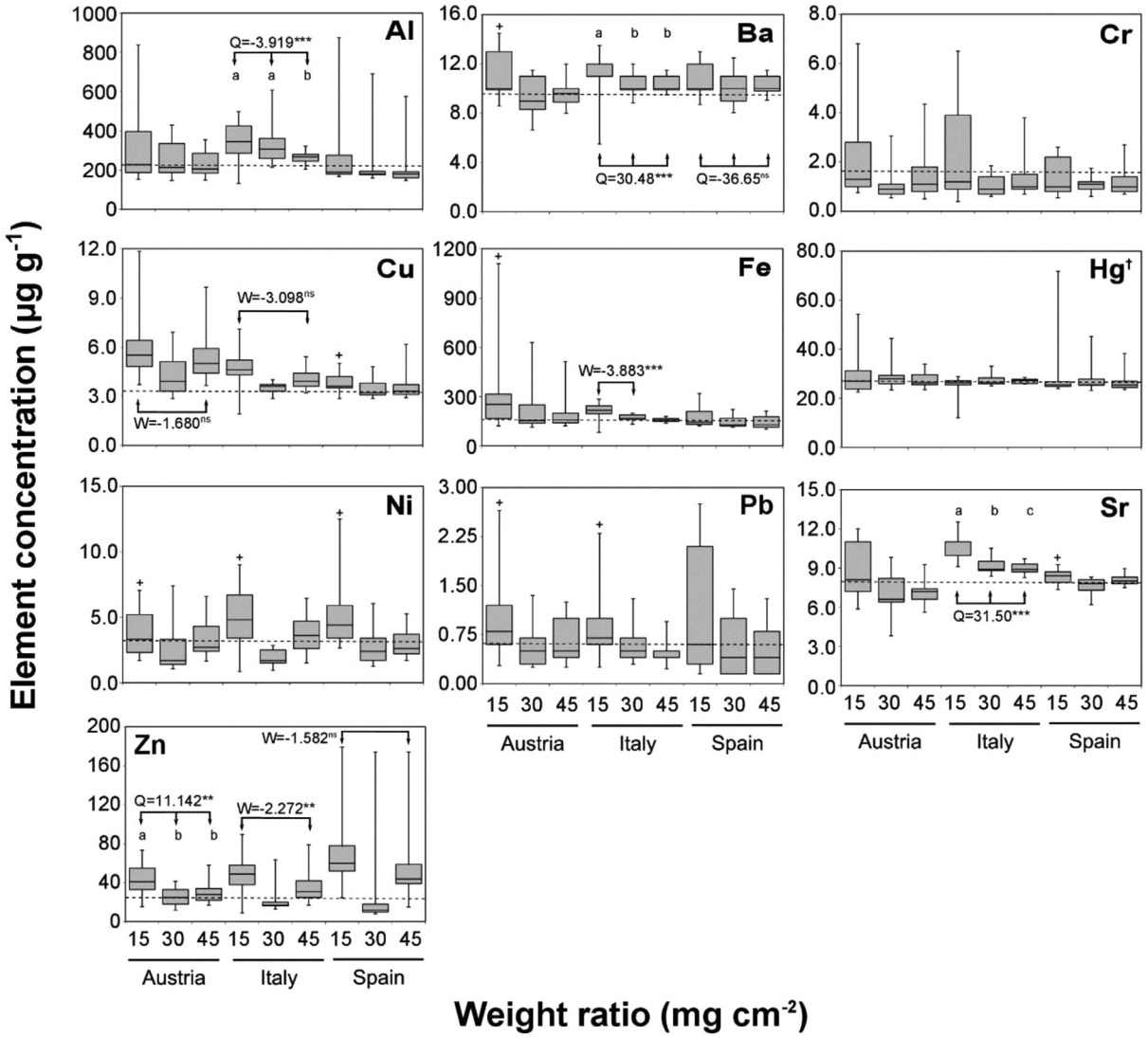

Fig. 5. Box-plots of the element concentrations (µg g^{-1}) in the moss exposed in Mosspheres prepared with different moss weight/sphere surface ratio (W15, W30 and W45) in Austria, Italy and Spain, see text for specifications. The dashed line represents the LOQ_T. BOX: inside band = median; extremities = 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; *p < 0.05, **p < 0.01, ***p < 0.001.

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

in part those of Zechmeister et al. (2006) and Ares et al. (2014): the maximum element interception is gained when the moss material is exposed to the air in a thin layer with all the shoots equally exposed, with no or scarce overlap among gametophores and without leaflets flattening. In particular, Ares et al. (2014), by using *Sphagnum denticulatum* Brid., observed a general increase in the moss post-exposure concentrations of Cd, Pb and Zn when they decreased the moss amount inside the bags.

Moreover the smallest ratio we applied (15 mg cm^{-2}), did not affect the data spread in a significant way, since it was already adequate to minimize replicability problems. Indeed, this ratio is 3 times higher than that suggested by Ares et al. (2014) as a compromise between pollution signal and data replicability. Therefore, we conclude that the quantity of moss contained within the Mossphere significantly affects the uptake performance of the device; in particular, the smaller the amount of moss, the better uptake performance, with no effect on the data spread, particularly when the ratio is not higher than 15 mg cm^{-2}. This is probably the result of a more homogenized distribution of elements on the surface of the receptor (moss), thereby minimizing analytical variation.

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 uptake 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 uptake 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 uptake rates for a higher number of elements. Nevertheless, the 12 week period was the only one showing the highest uptake rates for at least one element in all the countries. As in the previous experiments, no clear differences in terms of data spread were evidenced by the MAD/median ratio (Table 4).

In the moss bag technique the duration of the exposure period has been one of the most studied aspects (e.g. Gailey 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; Gailey and Lloyd, 1986c; Tavares and Vasconcelos, 1996; Vasconcelos and Tavares, 1998; Basile et al., 2008, 2009;

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

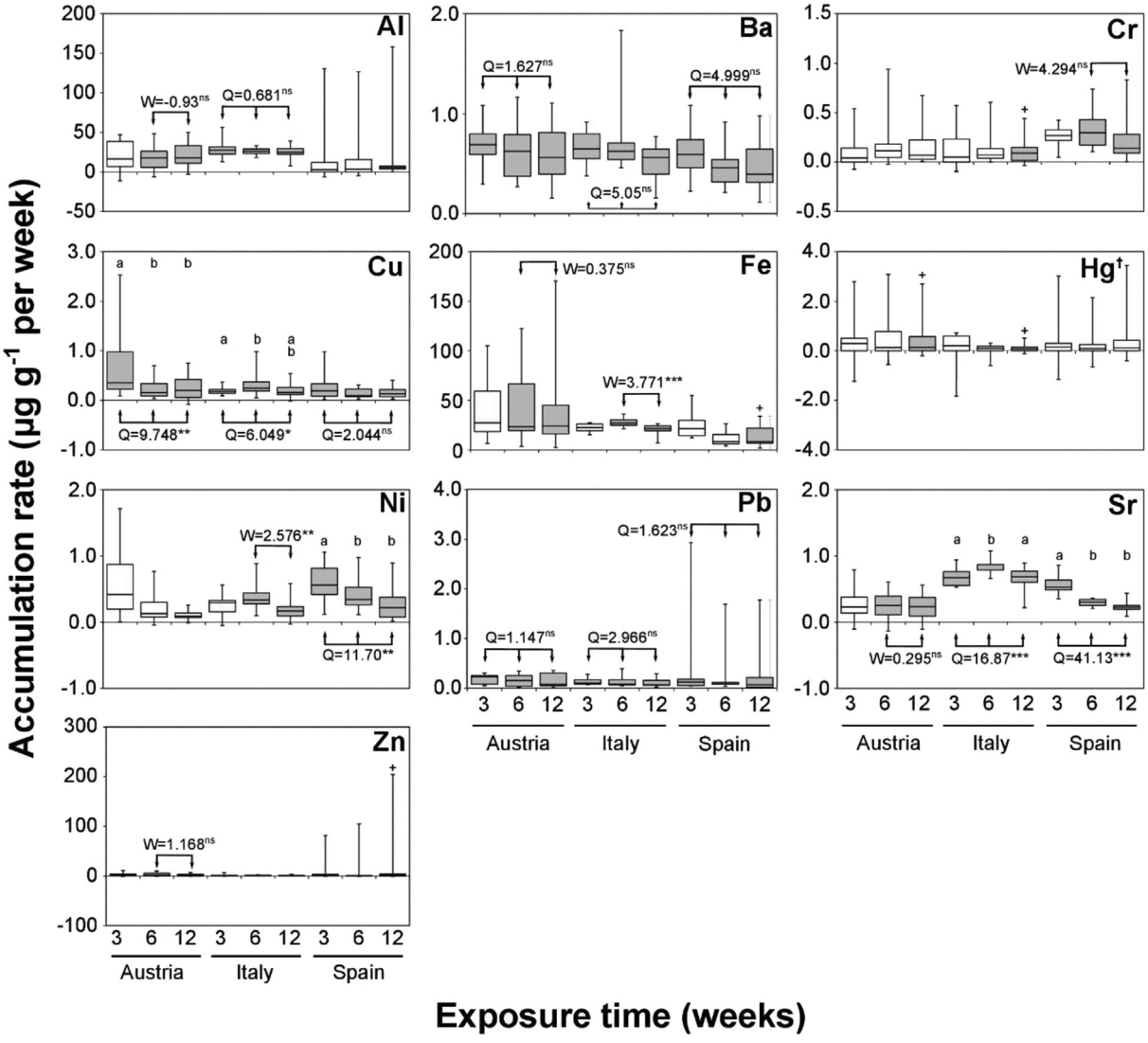

Fig. 6. Box-plots of the accumulation rate (μg g^{-1} per week) in the moss exposed in Mosspheres for 3, 6 and 12 weeks in Austria, Italy and Spain, see text for specifications. BOX: inside band = median; extremities = 1st and 3rd quartiles; whiskers = MIN and MAX. W, Q are the values of Wilcoxon and Friedman ANOVA tests. White boxes: values below the reference criterion; grey boxes: values above the criterion; "+" indicates the only option(s) fulfilling the criterion; *p < 0.05, **p < 0.01, ***p < 0.001.

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 uptake 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 uptake 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 for a period not shorter than 6 weeks because it ensures an adequate signal in terms of enrichment 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, shorter exposure periods could be enough.

3.6. Effect of the exposure height

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

The vertical profile of contamination is the result of the complex process of dispersion of contaminants in air masses and surface deposition, which is affected by factors related to atmospheric conditions or the type of distribution of contaminants (i.e. association with particles of different sizes or in gas phase), as well as aspects such as air turbulence, specific location of the source of emission (i.e. the vehicle exhaust pipes − fumes − and wear and tear of wheels and asphalt − particles and the emissions from domestic fuel) or topography of the area (i.e. presence of walls, buildings) (Aničić et al., 2009; Adamo et al., 2011; Vukovic 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 Vukovic 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; Thorpe and Harrison, 2008).

However, as for the other elements determined in the present work, most authors did not find any vertical patterns of moss bags concentrations in previous studies. Rivera et al. (2011) did not find any differences between Al, As, Cd, Cr, Cu, Mo, Pb, Sb, Sn and Zb concentrations in *Hylocomium splendens* bags exposed on balconies at heights of 3−21 m in Girona (NE Spain). De Nicola et al. (2013) concluded that there were no differences in Cd, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Pb and Zn concentrations in *Hypnum cupressiforme* transplants among samples exposed at heights of 3, 6 and 9 m in street canyons in Naples. In the same way, Vukovic 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 uptake performance (see sections 3.2 and 3.3) in each scenario. However, this was not true for the weight/surface ratio, which, according to this experiment, should be not higher than 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 sufficient element enrichment in 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 uptake 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 enrichment 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

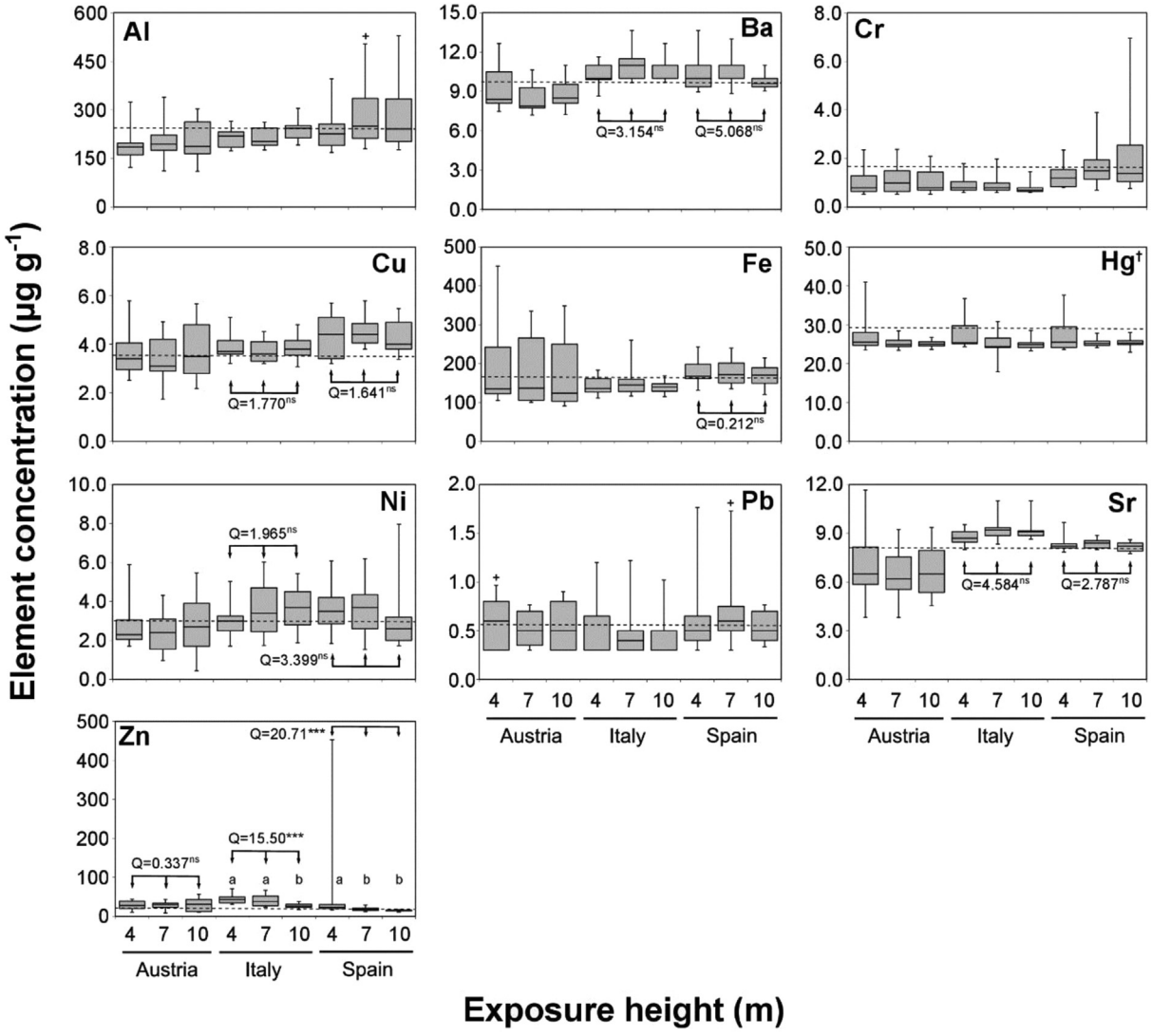

Fig. 7. Box-plots of element concentrations (µg g^{-1}) in the moss exposed in Mosspheres at 4, 7 and 10 m above the ground in Austria, Italy and Spain. The grey dashed line represents the LOQT. BOX: inside band = median; extremities = 1st and 3rd quartiles; whiskers = MIN and MAX. Q are the values of Friedman ANOVA test. "+" indicates the only option(s) fulfilling our criterion; *p < 0.05, **p < 0.01, ***p < 0.001.

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

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 m option reached the threshold. For the remaining comparisons, no differences in terms of uptake 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, 2014); 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 among the shape and the mesh size of the bags tested no one clearly enhance the uptake capacity of the

enclosed moss, as well as no differences were found among the moss exposed at the heights tested. 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 uptake signal in the majority of the sites, 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, we suggest the use of a Mossphere, because it is reusable, not "home-made" and with a regular and fixed shape; it should be prepared with 1 or 2 mm mesh net (to avoid the loss of material) and a moss content allowing a weight/surface ratio not higher than 15 mg cm^{-2} and it should be exposed at 4 m above the ground (this for practical reasons) for a period not shorter than 6 weeks.

To further reduce variability in the samples, to improve the sensitivity of the methodology 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.

Appendix A. Supplementary data

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