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Sphagnum palustre clone *vs* native *Pseudoscleropodium purum*: A first trial in the field to validate the future of the moss bag technique[☆]

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ABSTRACT

Although a large body of literature exists on the use of transplanted mosses for biomonitoring of air pollution, no article has addressed so far the use and the accumulation performance of a cloned moss for this purpose. In this work, a direct comparison of metal accumulation between bags filled with a *Sphagnum palustre* L. clone or with native *Pseudoscleropodium purum* Hedw., one of the most used moss species in biomonitoring surveys, was investigated. The test was performed in sites with different atmospheric contamination levels selected in urban, industrial, agricultural and background areas of Italy and Spain. Among the eighteen elements investigated, *S. palustre* was significantly enriched in 10 elements (Al, Ba, Cr, Cu, Fe, Hg, Pb, Sr, V and Zn), while *P. purum* was enriched only in 6 elements (Al, Ba, Cu, Hg, Pb and Sr), and had a consistently lower uptake capacity than *S. palustre*. The clone proved to be more sensitive in terms of metal uptake and showed a better performance as a bioaccumulator, providing a higher accumulation signal and allowing a finer distinction among the different land uses and levels of pollution. The excellent uptake performance of the *S. palustre* clone compared to the native *P. purum* and its low and stable baseline elemental content, evidenced in this work, are key features for the improvement of the moss bag approach and its large scale application.

1. Introduction

From the pioneering work of Goodman and Roberts (1971), the moss transplant technique has been largely employed all over the world. This methodology, using small nylon net envelopes containing transplanted moss and exposed in the environment, provides several advantages in terms of cost effectiveness and representativeness of data collected over territory. In the last decades, new outcomes on the presence and distribution of inorganic and organic pollutants in the air were reached from the application of the moss bag technique, confirming its usefulness, high sensitivity and versatility. In fact, using this technique, important information about air quality were obtained in surveys from urban environments, even city tunnels and street canyons (e.g. Aničić et al., 2009; Zechmeister et al., 2006a,b), as well as industrial sites (e.g. Fernández et al., 2000; Culicov et al., 2005). The moss bags proved to be a useful tool to implement the information derived from emission inventories (e.g. Iodice et al., 2016) and to validate particle dispersion predictive models (De Nicola et al., 2013). Moreover, they were successfully employed in order to study chemical properties of particulate matter (PM) from agricultural sites and marine aerosol (Adamo et al., 2011; Di Palma et al., 2017), and also to differentiate airborne pollutant sources in sites very close to each other (e.g. Capozzi et al., 2016a; Tretiach et al., 2011).

One of the main flaws of the moss bag approach is represented

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by the fact that all published results are generally comparable only within the same research settings due to the lack of a shared and standardized experimental protocol, from the choice of the moss species, the bag preparation, to the pre- and post-exposure treatments and the exposure conditions. Only recently the efforts of the researchers were focused on the implementation of these aspects. In particular the international team participating in the FP7 European project “MOSSclone” (www.mossclone.eu) aimed at the development and the optimization of a standardized protocol for the moss bag technique, in order to facilitate a direct comparison between different biomonitoring surveys. In the framework of this project *Sphagnum palustre* L. was selected among four moss species for its physical and chemical properties (González and Pokrovsky, 2014; González et al., 2016) and easy fast growth to be axenically cloned in bioreactors (Beike et al., 2015). This species was also characterized on a chemical and a molecular ground (Di Palma et al., 2016). The chemical analysis performed on different pre-treated moss materials always showed that the elemental content of the *S. palustre* clone is very low and stable in comparison to that of the conspecific, wild-growing moss. In addition, the clone proved to be also suitable to accumulate polycyclic aromatic hydrocarbons (PAHs) (Concha-Grana et al., 2015), so that an even larger number of pollutants can be evaluated with the same moss sample. Moreover, the variables affecting the exposure protocol were studied in detail by Capozzi et al. (2016b), evidencing that the moss density inside the bags and the exposure duration are the most influent variables for the accumulation capacity of the moss. Due to time constraints, this latter standardization assay was necessarily carried out in parallel with the development and the testing of the *S. palustre* clone. Since the massive collection of naturally growing *Sphagnum* species must be avoided for conservation purposes (92/43/EEC), we used the native moss *Pseudoscleropodium purum*, also frequently employed for biomonitoring purposes (e.g. Harmens et al., 2010; Ares et al., 2012). However, the *S. palustre* clone uptake capacity remained to be tested in a real biomonitoring survey. In this work a direct comparison of the performances of the *S. palustre* clone and the native *P. purum* is investigated for the first time on the basis of a bag exposure test performed in sites of Italy and Spain with different land uses. Since the baseline elemental content in the pre-exposed clone proved to be always lower and stable than in native mosses (Di Palma et al., 2016), we wanted to investigate if (*i*) the clone is more sensitive in terms of metal uptake (i.e., it is able to detect metals also in low polluted environments); (*ii*) the clone provides a higher signal showing a better performance as bioaccumulator compared to *P. purum*.

2. Materials and methods

2.1. Moss materials and experimental design

Sphagnum palustre was cloned and produced in a photobioreactor, as described in Beike et al. (2015) and Reski et al. (2016). *Pseudoscleropodium purum* was identified on the basis of the diagnostic morphological characters (Smith, 2004) with the aid of a stereomicroscope and a light microscope. The moss was collected in a background area of SE Galicia (NW Spain; 42°32′8.19″ N, 7°49′48.89″ W), and selected on the basis of previous results (Boquete et al., 2013). The two species were exposed in parallel in triplicate in 10 sites (i.e. 30 samples for each species) with different atmospheric contamination levels, chosen in urban, industrial agricultural and background areas of SW Italy and NW Spain (see Table 1 for details). Following the outcomes of the MOSSclone consortium, Mosspheres (spherical shape, moss weight/bag surface ratio of 10 mg cm^{-2}, 2 mm net mesh size, ø ~11 cm) were prepared with 3 g of the devitalized mosses. The *S. palustre* and *P. purum* bags were exposed coupled for six weeks, during spring 2014, at 4 m above the ground, using nylon strings linked to polyethylene or fibreglass rods. The bag characteristics and exposure procedure followed the protocol developed by the MOSSclone team, the only variable investigated in the present work is the uptake ability of the two moss species.

Table 1
Geographic coordinates for each exposure site and relative land use.

Country	Scenario	Coordinates	
		Latitude	Longitude
Italy	Urban	40°51′12.76″N	14°15′5.54″E
	Industrial-1	40°18′50.55″N	15°54′16.43″E
	Industrial-2	40°19′28.00″N	15°51′59.00″E
	Agricultural-1	40°18′57″N	15°57′15″E
	Agricultural-2	41° 1′16.74″N	14°15′21.20″E
	Background	40°51′49.53″N	14°15′16.11″E
Spain	Urban	42°52′54.58″N	8°32′22.77″W
	Industrial-1	43°41′44.93″N	7°28′20.20″W
	Agricultural-1	42°45′10.65″N	8°23′27.75″W
	Background	42°38′4.8″N	7°42′16.37″W

Although the EDTA washing is unnecessary for the clone, it is highly suggested for native moss materials (Di Palma et al., 2016); consequently, in order to reduce the variables likely affecting the experiment, both species were subjected to EDTA washing (further details on this point, and on the exposure protocol, see Capozzi et al., 2016a, 2016b; Di Palma et al., 2016).

2.2. Analytical determinations

The post-exposure moss material of each bag plus ten unexposed samples of each moss were oven-dried at 40 °C and separately homogenized in heavy metal-free mills (Retsch ZM 200) before any analytical determination. The concentrations of 19 elements, metals and metalloids (i.e. Al, As, Ba, Be, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb, Pd, Pt, Rh, Sn, Sr, V, Zn) 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. The inductively coupled plasma mass spectrometry (ICP-MS - Varian 820-MS) analysis was performed for all the elements except Hg at TE Labs (Tullow, Ireland) on filtered mineralized solutions of moss sub-samples of 0.5 g 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). In order to control contaminations during processing, analytical blanks (digestion solutions without mosses) were analyzed in parallel (one solution every 10 samples). For quality controls, replicates of the moss samples and the M3 certified reference material (*Pleurozium schreberi* moss; Steinnes et al., 1997) were processed, 1 every 10 samples. The concentrations of Be, Co, Pd, Pt, Rh and Sn were under detection limits in the reference material. The recovery of elements from the reference materials was not lower than 88% (Ba) and the relative standard deviation (RSD) was not higher than 17% (Cr), with the only exception for As (50%). All the results are expressed on a dry mass basis, with samples kept at 40 °C to constant weight. The Hg analysis was performed at the University of Santiago de Compostela on about 0.2 g of moss by a mercury elemental analyser (Milestone DMA 80). For the analytical control, the certified reference material M3 was analyzed every 13 samples. In addition, every 10 samples, one sample was re-analyzed to control the overall error, and procedural blanks were used to calculate the limit of quantification (LQ). The obtained recovery for the reference material was close to 103%, the error was 5% and the LQ was 0.5 ng g^{-1}.

2.3. Data processing

The limit of quantification of the technique (LOQ_T) was applied for the assessment of enrichment: $xC_i + 1.96sC_i$, 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). Comparisons between the two mosses were based on those elements showing concentrations higher than the LOQ_T in the arbitrarily fixed limit of 60% of all samples (i.e., 18 out of 30) for at least one moss species. All data were processed using Microsoft Excel and STATISTICA ver. 7. Since the distributions of the data were not normal ($p < 0.05$, Shapiro-Wilk test), non-parametric statistic tests were applied. The Spearman's Rank-Order Correlation and the Wilcoxon matched pairs test were used to evaluate respectively: the coherence of the information provided by the two bio-sensors and the significance of differences between their elemental uptakes.

3. Results and discussion

3.1. Pre-exposure moss content

Of the eighteen analyzed elements, Be, Cd, Co, Pd, Pt, Rh and Sn were below quantification limits (BQL) in both pre- and post-exposed mosses (C_0 and C_f, from now on, respectively). The pre-exposure values of the elements simultaneously present in both mosses, and hence useful for our comparisons, are shown in Table 2. Lead and V were BQL in both mosses; for these elements the QL/2 was used as pre-exposure value. The concentration of Al, Ba, Cr, Cu, Hg and Sr were always lower in *S. palustre;* the contents of the remaining elements were in the same range in the two mosses.

3.2. Post-exposure content in P. purum and S. palustre

In the post-exposed *P. purum* As, Cr, Fe, Ni, V and Zn did not meet the indicated 60% criterion; in particular, As and Ni were never taken up, Cr and Zn were found in less than 20% of the sites, while Fe and V were both accumulated in 47% of the sites. The percentage of accumulation (PoA) is reported in Table S1 (supplementary material) and was calculated as follows: $(C_f\text{-}C_0/C_0) \times 100$. The overall PoA ranged between -100% and 1100% (min-max) of the C_0 values, respectively for As (in almost all sites) and Pb for pair 28. The element showing the highest PoA median value was Pb with 67%. The PoA median over all sites and elements was 31%.

In the post-exposed *S. palustre* only As and Ni never satisfied the above criterion, being found accumulated in 33% and 0% of the exposure sites, respectively. The remaining elements were always significantly taken up: Al, Ba, Fe and Hg in 100% of the sites; Cr, Cu, Pb and V in more than 80% of the sites; Zn in 73% of the sites. The percentage of accumulation is shown in Table S1. The overall PoA ranged between -67% and 10 194% (min-max) of the C_0 values, respectively for As (in most of the sites) and Al (pair 7). The element showing the highest PoA median value was Al with 2966%. The PoA median, over all sites and elements was 228%.

Table 2
Mean values and standard deviations of element concentration expressed as $\mu g\ g^{-1}$ (except for Hg, $ng\ g^{-1}$) in the moss samples analyzed before exposure; $n = 10$ for both species. In Italic the QL/2.

	P. purum	*S. palustre*
Al	188.9 ± 17.8	16 ± 3.54
As	0.32 ± 0.08	0.3 ± 0.07
Ba	8.25 ± 0.67	0.65 ± 0.11
Cr	0.92 ± 0.37	0.52 ± 0.04
Cu	3.07 ± 0.22	1.94 ± 0.67
Fe	97.3 ± 31.21	108.35 ± 13.49
Hg	25.00 ± 1.26	2.46 ± 0.63
Ni	1.79 ± 0.66	2.25 ± 1.4
Pb	*0.3 ± 0.07*	*0.3 ± 0.07*
Sr	6.69 ± 0.59	2.2 ± 0.26
V	*0.25 ± 0.12*	*0.25 ± 0.12*
Zn	16.1 ± 4.09	17.05 ± 3.46

3.3. Comparison between the uptakes in the two mosses

It is worth to note that only few works have been 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); in particular, no research articles include the comparison between a cloned and a native moss.

S. palustre was significantly enriched in ten elements (Al, Ba, Cr, Cu, Fe, Hg, Pb, Sr, V and Zn) out of twelve, while *P. purum* was enriched only in six elements (Al, Ba, Cu, Hg, Pb and Sr), despite the pre-exposure EDTA washing. For the latter elements, enriched in both mosses, *S. palustre* always showed significantly higher accumulation ($p < 0.001$, except Cu $p < 0.01$) compared to *P. purum* (Fig. 1 and Table S1); similarly, *S. palustre* always showed an accumulation higher than *P. purum* for all remaining elements, included As, not significantly enriched (Fig. 1). As evidenced by the box-plots in Fig. 1, *S. palustre* showed a wider uptake range indicating a higher sensitivity. In addition, more robust accumulation signals were always provided by the clone with different intensity depending on the considered element. For example, Fe and V, with comparable LOQ_t values (Fig. 1), were enriched only in *S. palustre*. Lead, with the same LOQ_t for both pre-exposed mosses (Fig. 1) was enriched in both species, but *S. palustre* accumulated Pb 6 times more than *P. purum* (based on the ratio between PoA values, see Table S1). For Ba, where different LOQ_t values were observed in the two mosses (Fig. 1), an enrichment occurred in both species, but *S. palustre* accumulated 40 times more than *P. purum.* It is worth noting the case of Hg, showing different LOQ_t and concentration for the two mosses (*P. purum* > *S. palustre*), but significantly higher accumulation signal in *S. palustre* (Fig. 1 and Table S1). Plotting the element contents significantly enriched in both mosses and grouped by the different scenarios (Fig. 2), it is evident that the *S. palustre* clone showed a higher accumulation signal (i.e. higher distance of the histograms bar top from the respective LOQ_t line) in all land uses. Therefore, the clone proved to be able to detect trace elements even in low polluted sites enabling a better distinction among different land uses and levels of pollution. 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 ability, especially considering that the devitalization eliminates any metabolic contribution to the uptake. The *S. palustre* clone has a SSA of 28 ± 1 $m^2\ g^{-1}$ d. w., with 0.65 mmol g^{-1} proton binding sites (versus the 0.55 mmol g^{-1} in *P. purum*), mainly as carboxylic and phenolic groups (González and Pokrovsky 2014); in addition, the same authors found that *Sphagnum* sp. has the highest proton and metal adsorption capacity in comparison to *P. purum*, *Brachytecium rutabulum* and *Hypnum* sp. Moreover, the *Sphagnum* species display morphological features (i.e. hyalocysts, empty and dead cells with a variable number of pores) making this group of mosses particularly suitable for the outside and inside cell wall uptake of airborne particulate matter (e.g. Giordano et al., 2005; Vingiani et al., 2004, 2015; Spagnuolo

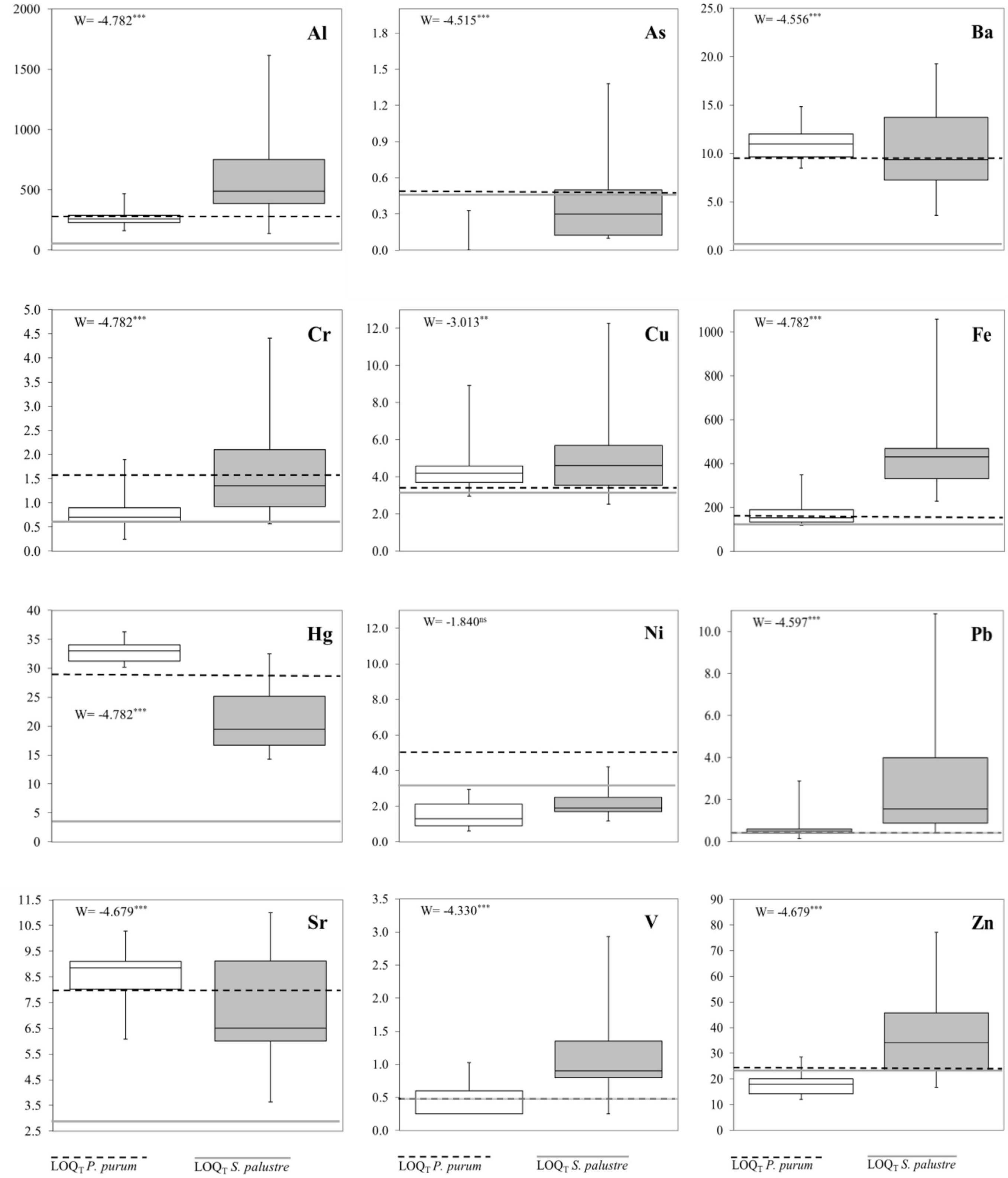

Fig. 1. Box-plots of element concentrations (μg g^{-1}, except for Hg ng g^{-1}) in the two mosses *P. purum* (white boxes) and *S. palustre* (grey boxes), n = 30. The dashed black line and the grey line represent the LOQ$_T$ for *P. purum* and *S. palustre*, respectively. BOX: inside band = median; extremities = 1st and 3rd quartiles; whiskers = MIN and MAX. The significance of the differences in the accumulation (i.e. accumulation: distance of the box-plots from the LOQ$_t$ lines), according to the Wilcoxon matched pairs test, is also reported. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

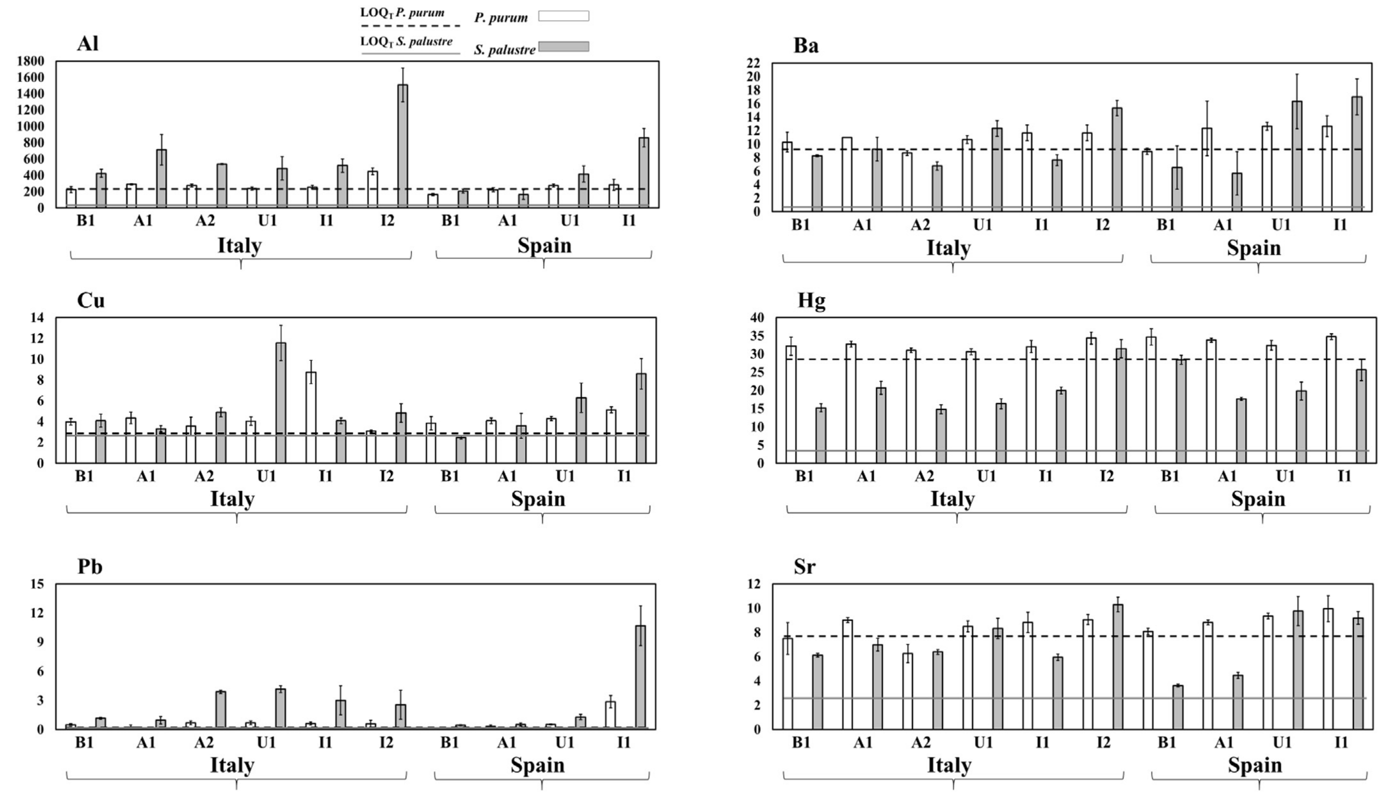

Fig. 2. Bar plots of the elements (mean content and standard deviation, n = 3) significantly enriched in both *P. purum* and *S. palustre* exposed in different scenarios (B: background; A: agricultural; U: urban; I: industrial). All the values are expressed in µg g^{-1}, except for Hg ng g^{-1}.

et al., 2017).

The coherence of information provided by the two bio-sensors, in terms of correlation between the same element content, is shown in Table 3. Among the elements fulfilling the adopted criterion, Al, Ba, Hg, Pb and Sr were significantly correlated between the two mosses; the only exception was Cu. Hence, the two species provided similar information considering the former five elements, but gave conflicting information for Cu. This contradiction could be explained considering the results provided by González and Pokrovsky (2014) and González et al. (2016), who described *Sphagnum* sp. as the most inert species in terms of biomass degradation and organic carbon leaching; in particular *S. palustre* and *Hypnum cupressiforme*, compared to *P. purum* and *Brachytecium rutabulum* (Hedw.) Schimp., were the most stable species in terms of Cu^{++} release. These characteristics could explain the higher uptake and retention capacity of Cu by *S. palustre* and justify the lack of correlation between copper contents.

Table 3
Spearman R correlation values between element accumulation (mean value) in the two mosses. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$., n = 30. In bold the elements fulfilling the fixed criterion in both moss species. In Italics, the elements not fulfilling the fixed criterion in both moss species (see M&M for details). For the remaining elements (Cr, Fe, V, Zn) *S. palustre* was the only respecting the criterion.

Al	**0.682*****
As	*0.006*^{ns}
Ba	**0.532****
Cr	0.282^{ns}
Cu	**0.044**^{ns}
Fe	0.468**
Ni	*0.572****
Hg	**0.522****
Pb	**0.789*****
Sr	**0.515****
V	0.499**
Zn	0.404*

4. Conclusions

The development of a clone produced in bioreactor represents a step ahead in the improvement of the moss bag approach. The clone is a homogeneous biomaterial that could be considered as a benchmark for this methodology. The low and stable baseline elemental content is a key feature for a large scale application of this technique. All the above results clearly indicate the better uptake ability of the *S. palustre* clone, in comparison with naturally grown *P. purum*, and its higher sensitivity both in giving a stronger accumulation signal and providing a finer distinction among pollution levels. This higher sensitivity enables the use of the clone in low impacted environments, or possibly for shorter exposure times (<6 weeks), although further tests are needed to verify this latter hypothesis. Besides, it represents a biomaterial bypassing the step of the moss collection and pre-exposure treatments; it is ready to use, and therefore can be easily employed by non-researcher personnel untrained in collection and identification of field mosses. We support the widespread application of a standardized moss bag protocol, in order to facilitate the direct comparison among different surveys, in the perspective of its inclusion among the monitoring technologies proposed by the EU legislation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2017.02.057.

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