Ecological Indicators 71 (2016) 388–397

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind

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

A. Di Palma[a], D. Crespo Pardo[b], V. Spagnuolo[b,*], P. Adamo[a], R. Bargagli[c], D. Cafasso[b], F. Capozzi[b,d], J.R. Aboal[e], A.G. González[f,g], O. Pokrovsky[g,h,i], A.K. Beike[j,k], R. Reski[k,l,m], M. Tretiach[d], Z. Varela[n], S. Giordano[b]

[a] Department of Agronomy, University of Naples Federico II, Via Università, 100, 80055 Portici (NA), Italy
[b] Department of Biology, University of Naples Federico II, Campus Monte S. Angelo, Via Cinthia 4, 80126 Naples, Italy
[c] Department of Physical Sciences, Earth and Environment, University of Siena, Via P.A. Mattioli 4, 53100 Siena, Italy
[d] Department of Life Sciences, University of Trieste, Via L. Giorgieri 10, , 34127, Trieste, Italy
[e] Department of Cellular Biology and Ecology, Faculty of Biology, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain
[f] Université de Bretagne Occidentale, LEMAR-UMR 6539, CNRS-UBO-IRD-IFREMER, Place Nicolas Copernic, 29280 Plouzané, France
[g] GET (Géosciences Environnement Toulouse) UMR 5563 CNRS, 14 Avenue Edouard Belin, 31400 Toulouse, France
[h] BIO-GEO-CLIM Laboratory, Tomsk State University, Tomsk, Russia
[i] Institute of Ecological Problems of the North, RAS, Arkhangelsk, Russia
[j] State Museum of Natural History, Rosenstein 1, 70191 Stuttgart, Germany
[k] Plant Biotechnology, Faculty of Biology, University of Freiburg, Schänzlestraße 1, 79104 Freiburg, Germany
[l] BIOSS—Centre for Biological Signalling Studies, 79104 Freiburg, Germany
[m] FRIAS—Freiburg Institute for Advanced Studies, 79104 Freiburg, Germany
[n] BIOVIA Consultor Ambiental, Edificio Emprendia, Campus Vida, 15782 Santiago de Compostela, Spain

ARTICLE INFO

Article history:
Received 18 January 2016
Received in revised form 21 June 2016
Accepted 23 June 2016
Available online 26 July 2016

Keywords:
Atmospheric pollution
Biomonitoring
Moss bags
Trace elements
DNA molecular markers

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 measured by ICP-MS analysis of 54 major and trace elements, with and without commonly applied pre-exposure treatments (oven devitalization and EDTA washing). Concentrations of almost all analyzed elements were significantly lower (from 10 to 100 times) in the clone than in conspecific field moss, apart from some elements (K, Mo, P and Na) deriving from the culture medium or EDTA treatment. Oven devitalization and EDTA washing did not significantly affect the clone composition. A comparison between the elemental composition of the clone with that of naturally growing *Sphagnum* species proved the particularly low elemental content of the clone. Therefore, in view of a rigorously standardized moss-bag protocol for the monitoring of persistent atmospheric pollutants, the use of the *S. palustre* clone, a biomaterial with very low and constant element composition, and homogenous morphological characteristics is strongly recommended.

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. A Clean Air Policy Package (CCEP-COM/2013/0918) was adopted in December 2013, with new air quality objectives up to 2030.

* Corresponding author.
E-mail address: valeria.spagnuolo@unina.it (V. Spagnuolo).

http://dx.doi.org/10.1016/j.ecolind.2016.06.044
1470-160X/

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 for air monitoring, also combined with automatic devices and emission inventories (Adamo et al., 2008a; Spagnuolo et al., 2013; Harmens et al., 2015; Iodice et al., 2016). The 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 can 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 of biomonitoring (Ares et al., 2012) and an essential prerequisite is the morphological and chemical homogeneity of the exposed material. In this sense, devitalizing and EDTA-washing treatments are recommended during moss preparation (Ares et al., 2014). Devitalization by oven drying prevents moss deterioration and enables the efficiency of contaminant capture to remain constant, as capture is mainly due to passive uptake processes that are independent of the vitality of the moss (Adamo et al., 2007; Giordano et al., 2009; Fernández et al., 2010). The use of chelating agents such as EDTA enhances the release of metals bound to cation exchange sites (Lodenius and Tulisalo, 1984). EDTA washing in moss transplants decreases element content in pre-exposure biomaterial, making it more sensitive to environmental pollution inputs (Iodice et al., 2016). 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 (i) the molecular and (ii) chemical signature of the *S. palustre* clone developed within the FP7 European project Mossclone. 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, such as oven devitalization and EDTA washing, and compared with that of naturally grown *Sphagnum* species.

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 of *S. palustre* (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 °C), followed by 44 cycles including the steps: denaturation at 94 °C for 1 min, annealing at 52 °C and 56 °C for 1 min for the primers ISJ 04 and ISJ 10, respectively, and elongation at 72 °C for 80 s. A further final extension at 72 °C for 5 min completed the PCR programme. 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 Biosystems); electropherograms were edited and aligned in Bioedit ver. 7.1 to obtain consensus sequences. The GenBank accession numbers of the sequences are KJ865421, KJ865420 and KJ865419, respectively.

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 Biosystems); fragment profile was visualized as an electropherogram by GeneMapper ver. 3.1 Software (Applied Biosystems).

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 electrophoresis on 1.5 agarose gels.

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 h (untreated clone, C-U) and after the following treatments: (1) EDTA washing and oven drying at 40 °C for 8 h (C-EDTA); (2) oven devitalization by consecutive 8 h-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; 1 l EDTA/12.5 g d.w. of moss) and 3 washes of 20 min each with distilled water (1 l 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 h (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 samples (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 min-holding stage at 150 °C, 1600 W and 100 psi was applied. After cooling, the mineralized solutions were evaporated at 70 °C for 24 h 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%).

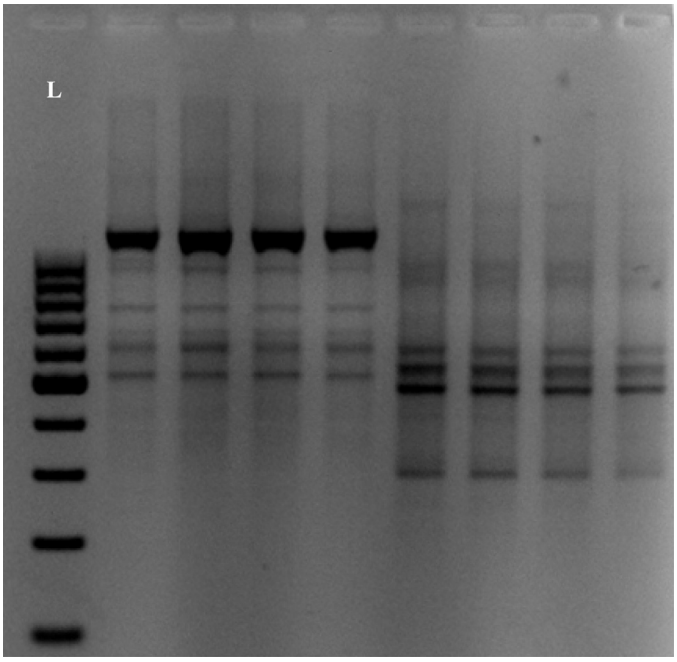

Fig. 1. RAPD amplification of the clone 12a; L = ladder 100 bp; lanes 2–5: DNA amplifications of 4 different shoots by OPB15 primer; lanes 6–9: DNA amplifications of 4 different shoots by OPJ19 primer.

2.3. 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.

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 (Fig. 1). The RAPD technique is generally considered poorly reliable because the shortness of the primers allows for the annealing to any DNA template eventually contaminating the sample. However, this problem is strongly reduced in axenic plant material; moreover, the used annealing temperature of 40 °C (i.e. 5 °C higher than that in the original amplification protocol proposed by Skotniki et al. (1999), greatly enhanced the stringency of the reaction and produced very constant banding patterns.

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

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

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

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

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

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

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

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

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

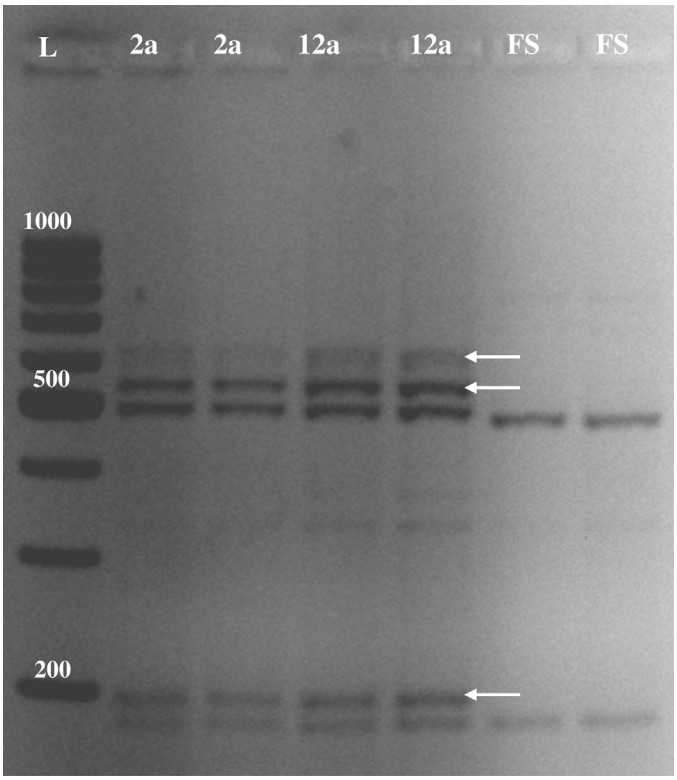

Fig. 2. Double digestion by Hind III/HinfI of RAPDf DNA region. L = ladder 100 bp; 2a and 12a are two different lines of *S. palustre* clone; FS = field shoots. Each digestion was performed on a different DNA extractions.

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 exploratory multivariate analysis of the element concentrations in the field (FS) and in the clone (C) *S. palustre* samples (see Table 4) revealed two main clusters (a and b) clearly separating them (Fig. 3). Both clusters were divided in sub-clusters generally according to the different treatments, even if the between-group variance was not significant.

In general, regardless of treatment, most element concentrations were significantly lower ($p < 0.05$) in the clone than in the field moss (Table 4). Indeed, in the latter the concentrations of Al, Ba, Cs, Hf, Nb, Pb, Ta, Y and rare earth elements were two orders

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
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

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 content of these elements in the clone tissues could interfere with the ability of the clone to monitor their occurrence in the environment, especially at low or very low pollution levels. Nevertheless, element concentration in the culture medium was established after several trials (Beike et al., 2015) and only further experiments might test the possibility to reduce them in the growing culture. Likely, an additional rinsing by distilled water might help to wash out the elements deriving from culture medium.

The high concentrations of some typical soil elements (i.e., Al, Fe, Ti, Ca and Mg) in the field samples would suggest the contribution of soil dust to the moss chemical composition (Bargagli, 1998; Adamo et al., 2008b).

Devitalization is a very useful option for biomonitoring with moss transplants 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; Capozzi et al., 2016). The oven devitalization, as well as the EDTA treatment, did not produce any substantial changes in the elemental composition of the moss samples; no statistically significant difference was observed between FS-U and FS-100, and between C-U and C-100 or in the

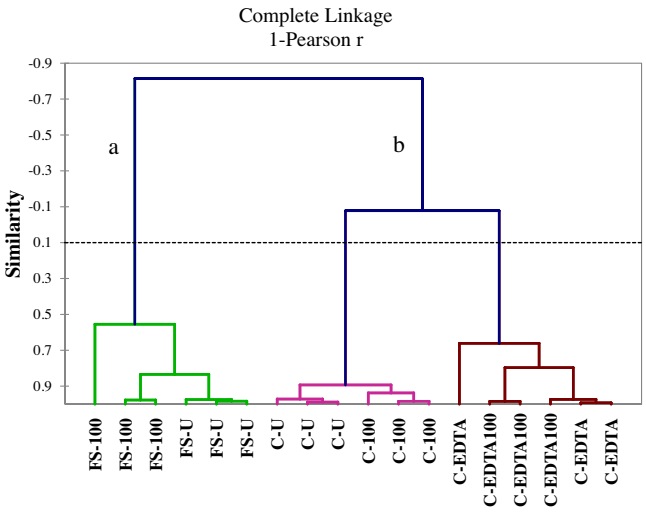

Fig. 3. Cluster Analysis of the element concentration data related to FS and C *Sphagnum* samples. For the labels see paragraph 2.2.1.

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, likely in virtue of the already very low element concentrations in the biomaterial. On the other hand, the EDTA washing added Na and induced morphological damages to the moss clone, making its shoots and leaves very fragile (see SEM micrographs in Fig. 4). On the base of these observations, we suggest to omit this treatment in the set-up of clone bags.

The comparison of the clone elemental composition with literature data for other *Sphagnum* species is made difficult by differences in sample preparation and chemical digestion procedures. Nevertheless, the treated and untreated clone samples had the same levels or even lower element concentrations than other *Sphagnum* species collected for biomonitoring purposes from reference areas of the world (Table 5). The only exceptions were *S. cristatum* from New Zealand (washed with conc. HNO_3 before the exposure; Archibold and Crisp, 1983), *S. olafii* from Greenland (moss species chosen to provide a baseline element content useful for estimating pollution levels in Europe, according to the UNECE ICP Vegetation monitoring programme; Zechmeister et al., 2011) and *S. girgensonii* from Russia (Aničić et al., 2009a,b). The acid washing as moss devitalizing treatment was criticized by recent studies (e.g. Adamo et al., 2007; Tretiach et al., 2007) recommending rather the use of other methods making treated moss shoots less fragile and consequently reducing the loss of material during the exposure.

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

4. Conclusions

Molecular analyses based on unilocus and multilocus DNA markers characterized a *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 monitoring of persistent air pollutants by moss-bags. 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". By virtue of its peculiar physicochemical properties (specific surface area, cationic exchange capacity, binding sites) henancing metal uptake capacity (González and Pokrovsky, 2014; González et al., 2016), this biomaterial proved an excellent biomonitor compared to field grown *Pseudoscleropodium purum* in a test with moss bags (unpublished results). 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.

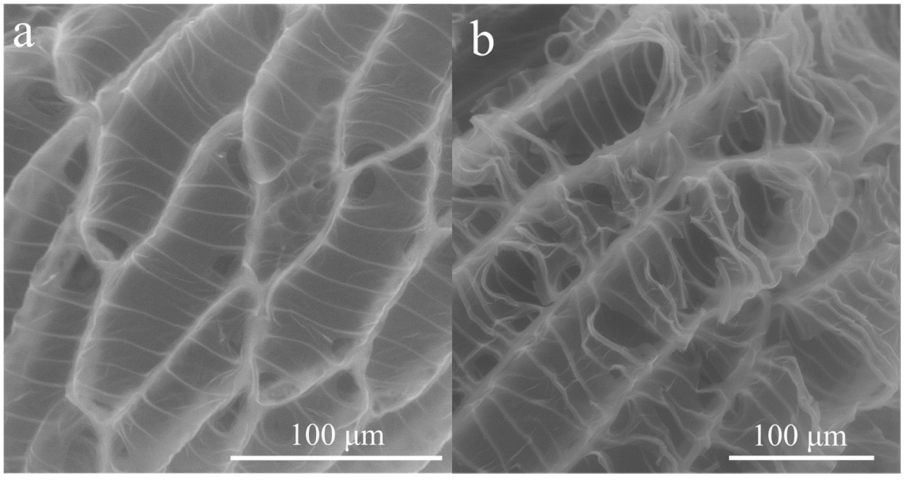

Fig. 4. Scanning electron micrographs of *S. palustre* clone before (a) and after EDTA treatment (b).

Table 5
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. (For interpretation of the references to colour in this table legend, the reader is referred to the web version of this article.)

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

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

Underlined: mean concentrations of a sample constituted by both *S. fuscum* and *S. tenellum*.
*Mean concentrations of a sample constituted by both *S. olafii* and *Aulacomnium turgidum*.
[1] Szczepaniak et al. (2007), [2] Zechmeister et al. (2011), [3] Archibold and Crisp (1983), [4] Aničić et al. (2008), [5] Aničić et al. (2009a), [6] Aničić et al. (2009b), [7] Kupiainen and Tervahattu (2004), [8] Culicov and Yurukova (2006), [9] Culicov et al. (2005), [10] Vuković et al. (2015), [11] Yurukova and Ganeva (1997), [12] Adamo et al. (2003), [13] Giordano et al. (2005), [14] Vingiani et al. (2015), [15] Archibold (1985), [16] Vasconcelos and Tavares (1998), [17] Goodarzi et al. (2002), [18] Calabrese et al. (2015), [19] Ratcliffe (1975), [20] Dmuchowski and Bytnerowicz (2009), [21] Dmuchowski et al. (2011), [22] Muskett (1976), [23] Al-Radady et al. (1994).

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., 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.

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.

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, 143–156.

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

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., 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.

Bargagli, 1998. Trace elements in terrestrial plants. In: 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 University Press, Cambridge, 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.

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, 1447–1455.

Capozzi, F., Giordano, S., Aboal, J.R., Adamo, P., Bargagli, R., Boquete, T., Di Palma, A., Real, C., Reski, R., Spagnuolo, V., Steinbauer, K., Tretiach, M., Varela, Z., Zechmeister, H., Fernandez, J.A., 2016. Best options for the exposure of traditional and innovative moss bags: a systematic evaluation in three European countries. Environ. Pollut. 214, 362–373.

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., Fernández, J.A., Aboal, J.R., Carballeira, A., 2004. Active biomonitoring of element uptake with terrestrial mosses: a comparison of bulk and dry deposition. Sci. Total Environ. 324, 211–222.

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.

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.

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.

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, 2798–2805.

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.

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. Monit. 4, 400–407.

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

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.

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

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.

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.

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

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.

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.

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 mosses (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., Area, 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.

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.

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, 380–391.

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.
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.
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., 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), 18956–18966.
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.
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.
Zechmeister, H.G., Dullinger, S., Koellensperger, G., Ertl, S., Lettner, C., Reiter, K., 2011. Do metal concentrations in moss from the Zackenberg area, Northeast Greenland, provide a baseline for monitoring? Environ. Sci. Pollut. Res. 18, 91–98.

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&9552;OJ:L:2002:067:0014:0030:EN:PDF
2004/107/EC:
http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri&9552;OJ:L:2005:023:0003:0016:EN:PDF
2008/50/EC:
http://eur-lex.europa.eu/legal-content/en/ALL/?uri&9552;CELEX:32008L0050
CCEP-COM/2013/0918:
(http://eur-lex.europa.eu/legal-content/EN/TXT/?uri&9552;CELEX:52013DC0918).