Highlights

- Particles of different composition are deposited on roadside moss samples.
- Washing step is mandatory to study the bioconcentration of PAHs in moss tissue.
- SEM-EDS allows to check the effectiveness in the removal of particles from sample.
- High molecular weight PAHs might be efficiently bioconcentrated in moss tissue.

*Revised manuscript with no changes marked
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Sample pretreatment to differentiate between bioconcentration and atmospheric depositionof polycyclic aromatic hydrocarbonsin mosses.

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Abstract

In this first approacha comparison using different sample pretreatment methodologies has been made to differentiate between total atmospheric deposition and bioconcentration of polycyclic aromatic hydrocarbons (PAHs) in moss samples (*Brachythecium rutabulum*). Samples were collected in a densely polluted urban area in Barakaldo (Biscay, Basque Country) andsubmitted to different cleaning procedures with the aim to remove as many deposited atmospheric particles as possible. Analysis by means of Scanning Electron Microscopy coupled to Energy Dispersive Spectroscopy (SEM-EDS) allowed to quantify the removal efficiency of each cleaning procedure and to chemically characterise particles still present in the pre-cleaned sample. Cleaning moss samples twice with deionised water in an ultrasound bath showed up as the most suitable way to remove solid particles deposited on their surface. Discerning between bioconcentration and atmospheric deposition is therefore possible after GC-MS quantitative analysis of non-washed and washed moss samples.

Keywords: moss; atmosphere; bioconcentration; SEM; PAH.

1. Introduction

Vegetation samples, like mosses and lichens, have been widely used to monitor and identify the sources of a wide range of pollutants like heavy metals (eg. Fernández et al., 2004; Giordano et al., 2005; Anicic et al., 2009) and persistent organic pollutants (POPs) (Blasco et al., 2006; Gerdol et al., 2002; Ötvös et al., 2004). Recent studies have started wondering if the cleaning step prior to the analysis of these samples should be carried out or not (Aboal et al., 2011; Spagnuolo et al., 2013). Still, these studies have only taken into account the effects of the washing step when determining heavy metals but not POPs.Since moss tissues have shown to retain atmospherically deposited polycyclic aromatic hydrocarbons (PAHs) as

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efficiently as trace metals (Milukaite, 1998), a larger research including the effects of the cleaning step during the analysis of POPs in moss and lichens is required.

As stated by Harmens et al (2008), when determining metallic deposition, samples should not be washed. On the other hand, if the aim is to determine the bioconcentration of pollutantsin these organisms and the related toxicity, samples should be free of any deposited particle and, therefore, cleaned prior to analysis. However, special care must be taken when washing vegetation samples in order to avoid any kind of contamination and/or non-desired extraction of the analytes from the matrix. Here, once again, only information for heavy metals has been reported (Pérez-Llamazares et al., 2011; Sentenac and Grignon, 1981; Spagnuolo et al., 2013).

Regarding the analytes to be studied in this work, PAHs are a large group of organic compounds with two ormore fused aromatic rings.Most of the PAHs with low vapour pressure in the air are adsorbed on particles, and as they are highly lipophilic chemicals, they have a relatively low solubility in water (Bjorseth and Ramdahl, 1985). Therefore,fewer problems are to be expected during the washing procedure when using water or other polar solvents compared to the case of heavy metals.

In this study mosses (*Brachythecium rutabulum*) were collected in a densely polluted urban area near a highway in Barakaldo (Biscay, Basque Country) and were submitted to different cleaning procedures before analysing them by means of Scanning Electron Microscopy coupled to Energy Dispersive Spectroscopy (SEM-EDS) to i) study the effectiveness of each procedure to remove solid particles deposited on the surface of the sample, ii)characterise the different particles observed in the sampleand iii) select the most appropriate pretreatment to discern between deposited and bioconcentrated organic pollution in moss samples.Washed (using the selected cleaning procedure) and non-washed samples were further analysed by Gas Chromatography-Mass Spectroscopy (GC-MS) to investigate the differences in PAH concentrations.

2. Materials and Methods

2.1. Sampling

- The moss species *Brachythecium rutabulum (Hewd.) Schimp* was selected for this study based on its abundance in the sampling area and not on its usage for biomonitoring studies in the surroundings as this was only a first approach to study the aforementioned hypothesis. Still, it has been reported to act as a good bioindicator in the literature (Ötvös et al., 2003; Samecka-Cymerman et al., 2009; Spirić et al., 2014) as it occurs in a wide range of habitats, and is especially common on wood and stones.

Moss samples were collected in a densely populated urban area near a heavy traffic area (Diputación Foral de Bizkaia, 2012) including a motorway (A-8, 78,000 vehicles day^{-1}) and two highroads (N-634 and N-637 with 10,000 and 134,000 vehicles day^{-1}, respectively) in Barakaldo (Biscay, Basque Country). The collection of samples was carried out in November 2011 after several days without rain in order to obtain the largest amount of deposited particles as possible on the sample. Samples were collected in an area of 20 m^2 using a scalpel and stored in plastic zipper bags inside portable coolers at low temperature for their transportation to the laboratory. Special care was taken to only collect mosses located on asphalt or cement, but not on soil, to avoid possible blending between soil particles and moss tissues during sample transport.

2.2. Cleaning procedures

In the laboratory samples were mixed to further separate them into 8 subsamples. In each subsample the apical segments (10-30 mm long) were cut from moss shoots and 20-30 of them were stored inside glass flasks. 7 different cleaning procedures (Table 1) were proposed with the aim of removing as many particles as possible from the surface of the sample.6 subsamples were submitted to a combination of magnetic stirring or sonication (400 W, ultrasonic bath, JP Selecta) together with 10 mL of Milli-Q water (Millipore, Carrigtwohill, Ireland) or acidified water (HNO_3 10%,Tracepur grade, Merck, Darmstadt, Germany) for different time lengths as mentioned elsewhere (Spagnuolo et al., 2013). Another subsample was put under a 0.15 bar stream of N_2 (99,9992%, Carburos Metálicos, Spain) as suggested by Ducceschi et al (1999), holding each shoot individually with tweezers to allow it face the stream from every direction. The remaining subsample was not cleaned and was treated as a control.

One of the major drawbacks when dealing with bryophytes and heavy metals consists on the length of the washing step (Sentenac and Grignon, 1981; Wells and Brown, 1990), as procedures of more than 30 s may alter the equilibrium of the extracellular cations. However, when dealing with non-polar organic compounds such as PAHs, the risk of changing this equilibrium does not end up being critical as the octanol-water partition coefficients (K_{ow}) for these organic molecules are rather high. Therefore, the chosen times for both sonication and agitation were considerably higher in some cases.

2.3. Scanning Electron Microscopy-Energy Dispersive Spectroscopy (SEM-EDS)

Prior to SEM-EDS analysis of the 8 subsamples, the already cleaned apical segments were dried in an oven at 50ºC (it has to be borne in mind that during the drying of samples some of the particles could be detached from the surface). Subsequently, subsamples were carefully mounted on an aluminium stub and coated with graphite.

Particles deposited on both abaxial and adaxial surfaces of the samples were observed at ambient temperature using a scanning electron microscope (Carl Zeiss AG, EVO 40, Oberkochen, Germany) equipped with an energy dispersive spectrometer (SDD X-max 50, Oxford Instruments, Abingdom, UK). SEM observations were carried out at magnificationsup to 2000x approximately, while the electron beam energy was fixed at about 20 eV, being the working distance in most of the cases 10 mm and the probe current of 100 pÅ. Particles were observed as backscattered electron images and further subdivided in three size classes (<2.5 μm, 2.5–10 μm and >10μm in maximum diameter) for their quantification using an image editing software (Adobe Photoshop CS5).

2.4. PAHs determination in moss

The concentrations of 16 selected PAHs were measured in washed (using the proposed procedure) and non-washed moss samples. Samples were freeze dried in a Cryodos apparatus (48 h, -52.2 ºC, $5.4 \cdot 10^{-2}$ mbar; Telstar, Spain) before analysis. All solvents used were of HPLC grade and were obtained from Lab-Scan (Gliwice, Poland). Approximately 1.5g of dried moss were accurately weighed in a Mettler AJ150 analytical balance (Mettler Toledo, Spain) and submitted to Microwave Assisted Extraction (MAE) together with a mixture of 20 mL of acetone:n-hexane (1:1) and 20 μL of deuterated PAH (S-4124 deuterated PAH mixture, Chiron AS. Trondheim, Norway) used as surrogate. The extraction conditions in the microwave (MarsXpress, CEM, Kamp-Lintfort, Germany) were as follows: for the first 5 minutes, the system was allowed to reach 120ºC using full power (100%, 1600 W); in the next 10 minutes, the temperature previously reached was kept constant.The extracts were filtered through Millex® HV0.45 μm PVDF filters (Millipore, Carrigtwohill, Ireland) and then concentrated to 1 mL using a gentle stream of nitrogen in a TurboVap LV Evaporator (Zymark, Hopkinton, MA, USA) at 35 ºC.A clean-up step was carried out by Solid Phase Extraction (SPE) with 2g Florisil® cartridges (purchased from Supelco) previously conditioned with 10 mL of n-hexane. Analytes were eluted with 14 mL of a mixture of n-hexane:toluene 65:35 (v:v). Subsequently, the extract was evaporated to dryness, re-dissolved in 100 μL of n-hexane and kept in dark at -18ºC until GC-MS analysis according to the procedure described elsewhere (Bustamante et al., 2010).

3. Results and discussion

3.1. Cleaning efficiency

A selection of the most representative SEM images obtained for all the subsamples after going through the different cleaning procedures are shown in Fig.1.

Dry deposition depends on physical characteristics of the particles (such as size and shape), the meteorological conditions (wind speed, thermal stability…), and morphological characteristics of the biological surface (Tomasevic et al., 2005).The quantification of the total amount of particles in all the obtained microphotographs and taking average values for each washing method led us to the next most important conclusions: as expected, subsamples subjected to different cleaning procedures show different amounts of remaining particles (b, 21; c, 44; d, 5; e, 5; f, 29; g, 31; h, 76; on average) but always less compared to the unwashed one (a, 91; on average). Procedures may be sorted according to their efficiency to remove particles (from lower to higher efficiency) as follows: h << c ~ b < g ~ f < d ~ e.

The use of a controlled stream of dry nitrogen (h) allowed the elimination of the largest particles (>10 μm) without damaging the structure of the matrix; however, like in the case of agitation (b and c), it resulted in an inefficient technique for the cleaning the smallest particles (<2.5 and 2.5-10 μm) on the samples (h, 72.4% and 27.6%; b, 72.7% and 18.2%; c, 70.4% and 25%, respectively). The ultrasonic bath assisted cleaning appeared to be the best way to obtain a particle-free surface (microphotographs d, e, f and g). Moreover, repeating the cleaning procedure twice (2x15 min) was more efficient than a single ultrasound run, regardless of using nitric acid or milliQ water, (microphotographs d and e vs. f and g). However, as the differences between d) and e) were not importantand considering other factors like ease of use, safety and that H_2O is less aggressive than HNO_3, we recommend to clean moss samples twice with deionised water in an ultrasound bath as the most suitable way to remove solid particles deposited on their surface. It has to be considered that these results might vary between species and direct extrapolations are to be avoided.

3.2. Characterisation of the particles

The potential of the SEM-EDS technique to characterise solid particles present in the surface of vegetation samples like moss using the elemental information provided in the corresponding XRF spectra was also tested. Several particles (found in a wide range of diameters up to 10 μm) in the microphotographs were randomly selected, and their XRF

spectra was measured. The chemical elements identified in the particles were C, O, Na, Cl, Mg, Al, Si, P, S, K, Ca, Fe and Zn, being C, O, Si, Ca and Al the most abundant ones (Fig. 2). Depending on their chemical composition, two categories of particles were observed: particles of biological sources such as those coming from moss itself, soot and pollen (Fig. 2. a, b and c), and particles coming from the re-deposition of atmospheric dust (Fig. 2. d, e and f). The particle in Fig. 2a was identified as part of the moss subsample as its spectra showed no difference compared to other studied areas in clean moss subsamples (C:O proportion 70:20, approximately). Therefore, this spectrum was treated as a blank when studying other foreign particles in moss subsamples.

Different chemical ratios were found in other subsamples. In Fig. 2b, a major abundance of C was observed compared to the moss blank spectrum (Fig. 2a), suggesting the presence of soot, an organic material typically found in the environment due to the incomplete combustion of wood or coal and a potential source of PAHs. Fig. 2c showed a similar spectrum to the blank one, slightly varying the proportion between C and O. This new particle, due to its form and composition, seemed to be a pollen particle, with a size of about 10 μm, and clearly differed from the previously studied soot particle.

Studying particles coming from the re-deposition of atmospheric dust, the presence of Ca in Fig. 2d was really notorious. This may suggest the presence of $CaCO_3$ in the particles attached to the moss samples, due to their proximity to the soil. Moreover, the spectrum in Fig 2e, indicates the presence, among others, of Al, Si and Fe. This kind of chemical elements can form diverse chemical structures, such as iron oxides and aluminosilicates.

Additionally, a glass particle was found in Fig. 2f, surely made up by Si, $CaCO_3$ and Na_2CO_3. It is important to highlight the presence of Mg in this microphotograph, as Mg can be used as an additive for colouring glass.

3.3. Deposition vs. bioconcentration

16 PAHs were analysed in two moss subsamples.One of them was washed using the chosen cleaning procedure (Table 1, procedure 4). The other one was directly analysed, without any prior cleaning procedure. The results are shown in Fig. 3. The already washed subsample was subjected to a second cleaning cycle in order to assure that the effects of the ultrasonic energy, using deionised water in the cleaning process, were not significant concerning the extraction of the bioconcentrated analytes in mosses (α=0.05, n=3).

As it can be observed, concentrations of most of the PAHs were lower in the previously washed subsample. However, in analytes like acenaphthylene and the heaviest compounds

such as benzo[k]fluoranthene, indeno[1,2,3-cd]pyrene, dibenz[ah]anthracene and benzo[ghi]perylene, no difference could be observed between the washed and non-washed subsamples (α=0.05). This fact suggests that even if this kind of compounds is mainly found associated to particles rather than to the gas phase, they can be easily bioconcentrated in the moss tissue.

PAH concentration in mosses and in particles attached to their surface has been previously reported to be related to the emission source (Ares et al., 2009; Augusto et al., 2009). In this case, mosses were collected close to a densely populated urban area near a heavy traffic area. Thus, a strong connection between these sources and the concentration of PAHs in both washed and non-washed samples was to be expected. Ratios between specific compounds, such as B[a]A/(B[a]A + Cry) and B[a]P/B[ghi]P have been proposed as valuable source indicators of traffic related sources for PAHs (Tobiszewski and Namiesnik, 2012). The results in this study, even though they are not representative, show a clear connection with traffic related sources not only in the case of non-washed mosses (B[a]A/(B[a]A + Cry) = 0.48; >0.35, vehicular emission; and B[a]P/B[ghi]P = 0.62; >0.6, traffic emission), but also in the case of the washed ones (B[a]A/(B[a]A + Cry) = 0.51; >0.35, vehicular emission), suggesting a potential link between the source of the PAHs found in particles attached to mosses and of those bioconcentrated in their tissues.

4. Conclusions

This preliminary research confirmsthat mosses may act as good sentinels of atmospheric pollution caused by PAHs. However, an appropriate sample pretreatment must be selected depending on the aim of the work. For the specie under analysis a previous washing step using ultrasonic energy and deionised water is mandatory if the objective is to remove as many attached particles as possible and to study the bioconcentration of PAHs in moss tissue. Still, a deeper research is necessary including other moss species and different sampling areas to reaffirm this statement.

During this approach SEM-EDS has been confirmed to be an attractive alternative to check the effectiveness in the removal of particles from the surface of the sample using different cleaning procedures. This technique also entails the characterisation of the particles present in the sample, which helps to identify the presence of potentially interfering motes (i.e. soot) in the analysis of PAHs. Finally, even though this observation deserves further investigation, the results obtained here suggest that i) high molecular weight PAHs could be efficiently bioconcentrated in moss tissue even if they are primarily adsorbed in atmospheric particles

rather than in gas phase and ii) there might be a potential link between the source of the PAHs found in particles attached to mosses and that of PAHs bioaccumulated in their tissues.

5. Acknowledgements

This work has been financially supported by the SUDOE Interreg IV B programme through the ORQUE SUDOE (Ref. SOE3/P2/F591/5) project, and the Working Community of the Pyrenees through the CTP 2010 P03 project. J. Bustamante is grateful to the UPV/EHU for his pre-doctoral fellowship.

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Table
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Tables

Table 1. Different cleaning procedures proposed for the 8 moss subsamples.

Subsample	Cleaning procedure	Time (min)
1	No cleaning	-
2	HNO_3 (10%) + agitation	240
3	Milli-Q water + agitation	240
4	Milli-Q water + ultrasonic bath	2x (15)
5	HNO_3 (10%) + ultrasonic bath	2x (15)
6	Milli-Q water + ultrasonic bath	15
7	HNO_3 (10%) + ultrasonic bath	15
8	Stream of N_2	5

Figure 1

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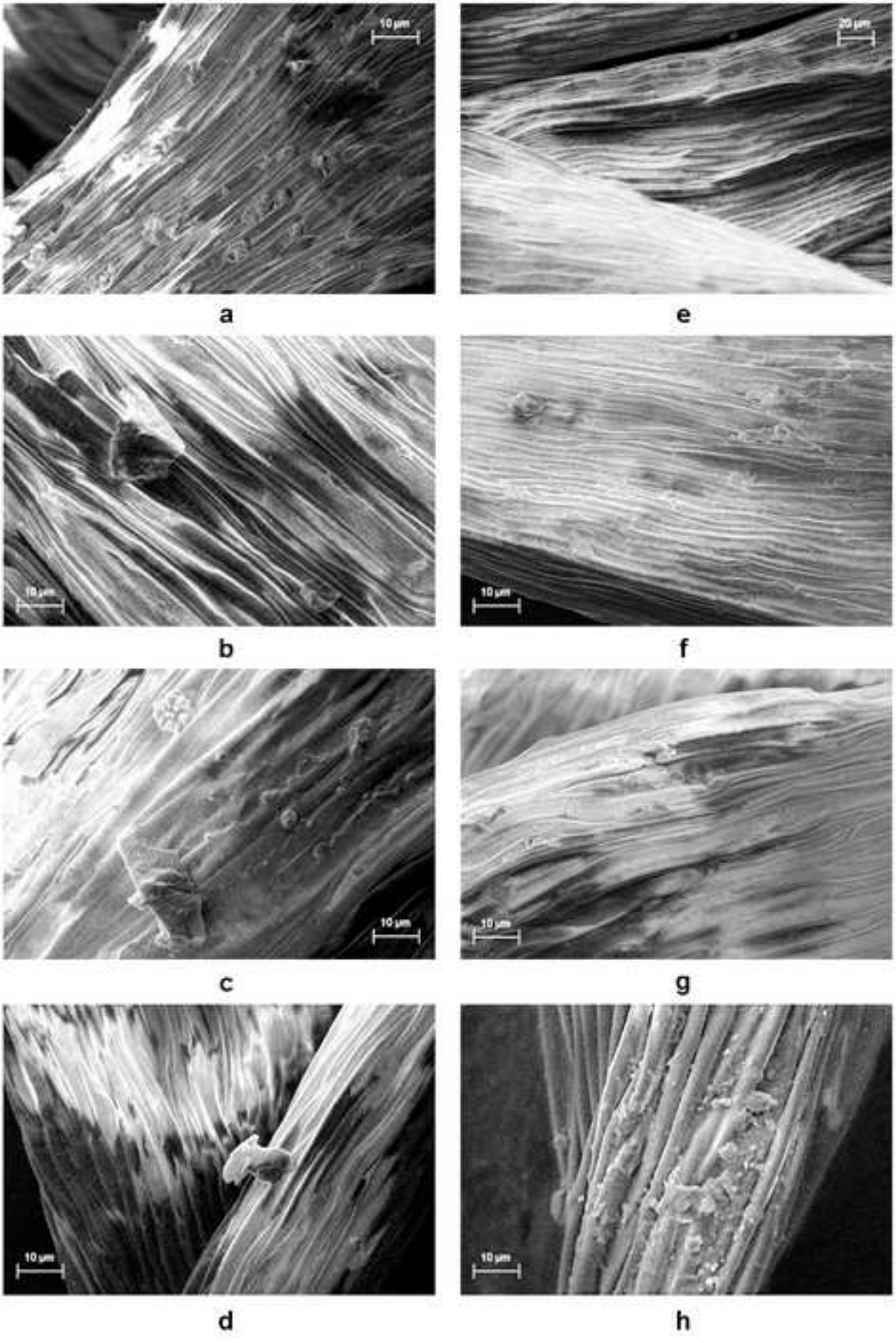

Figure 2

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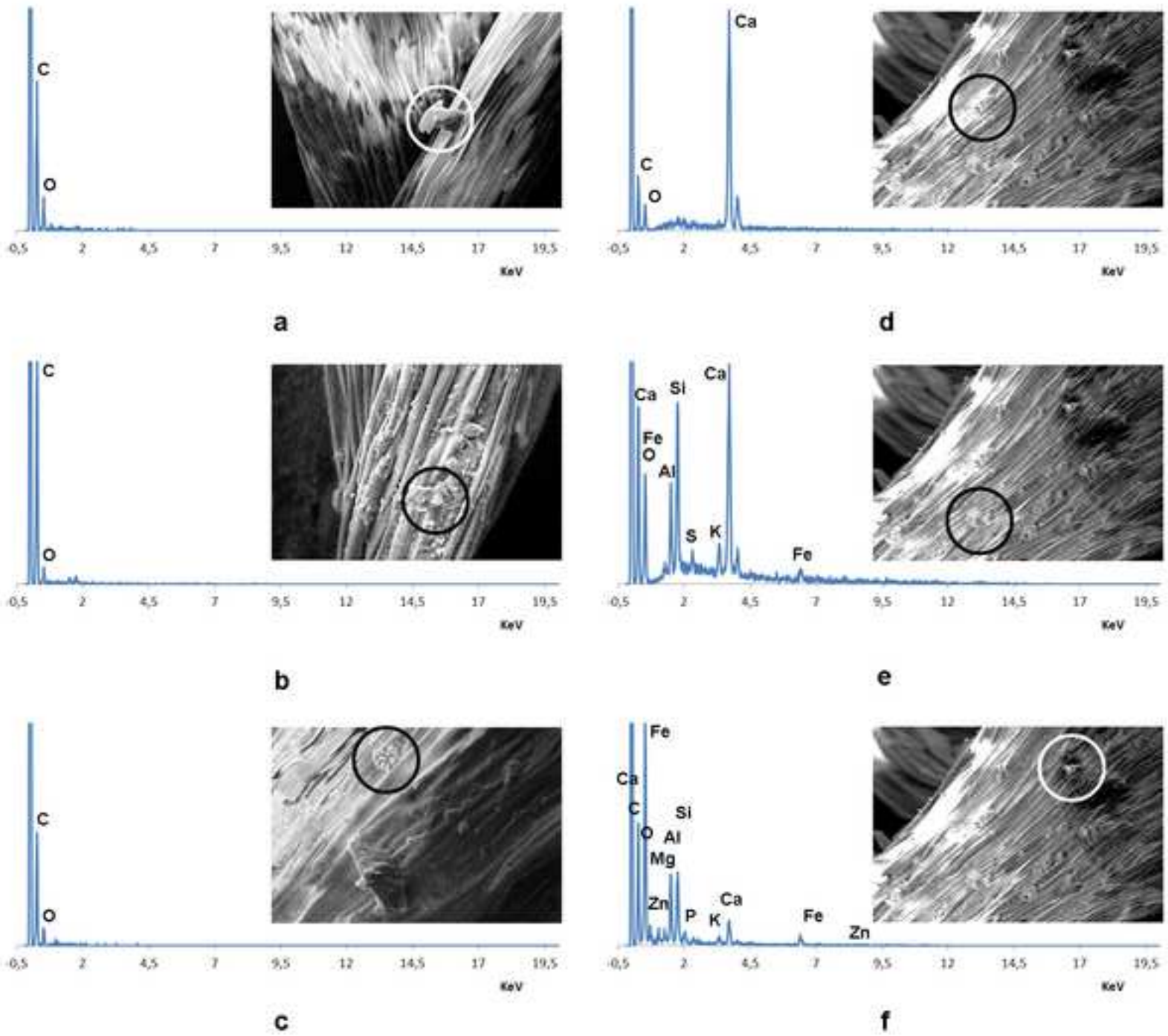

Figure 3

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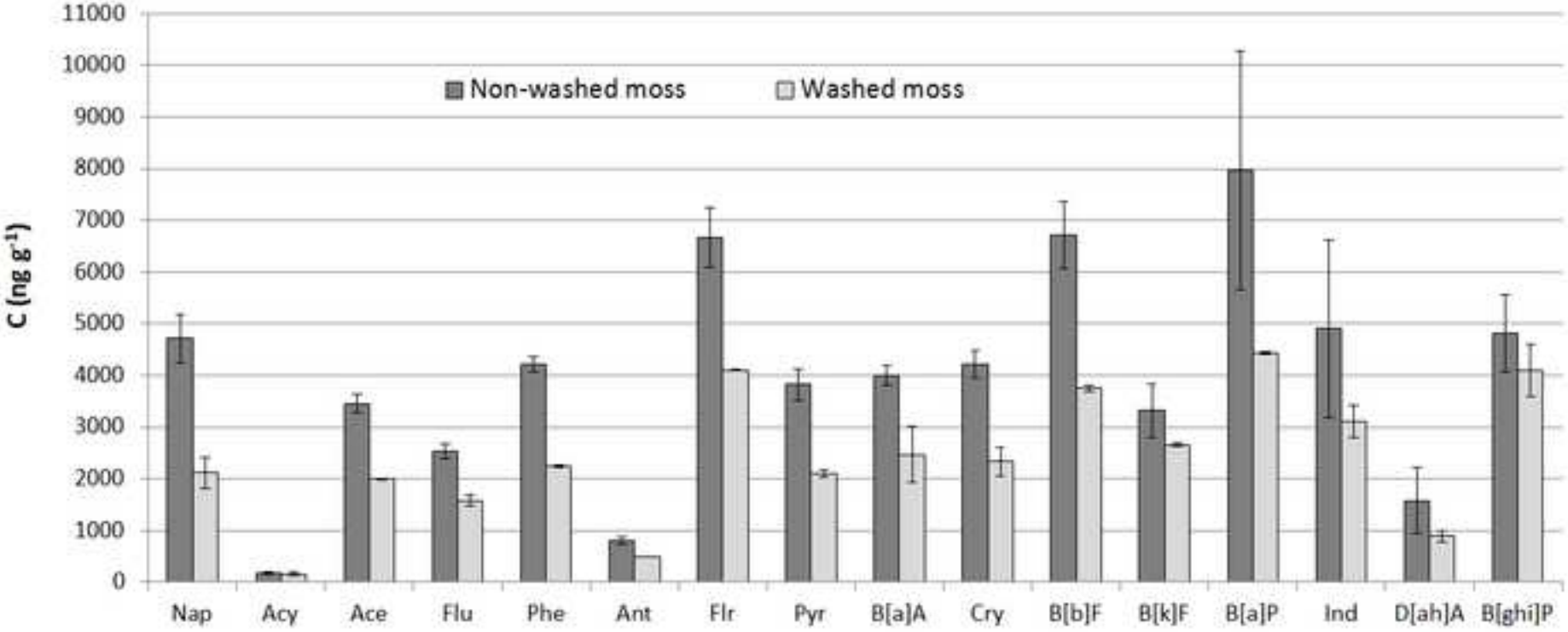

Figure captions

Fig. 1. Microphotographs showing particles on the surface of the unwashed moss subsample a) and the washed moss subsamples: b) 10% HNO_3 + agitation (240 min); c) H_2O + agitation (240 min); d) H_2O + USB (15 min x2); e) 10% HNO_3 + USB (15 min x2); f) H_2O + USB (15 min); g) 10% HNO_3 + USB (15 min); h) N_2stream).

Fig. 2. XRF spectra of particles present in moss subsamples. The particle corresponding to each spectrum is indicated inside a black/white circle.

Fig. 3. Concentrations (ng g^{-1}) of 16 PAHs including Naphthalene, Nap; Acenaphthylene, Acy; Acenaphthene, Ace; Fluorene, Flu; Phenanthrene, Phe; Anthracene, Ant; Fluoranthene, Flr; Pyrene, Pyr; Benz[a]anthracene, B[a]A; Chrysene, Cry; Benzo[b]fluoranthene, B[b]F; Benzo[k]fluoranthene, B[k]F; Benzo[a]pyrene; B[a]P; Indeno[1,2,3-cd]pyrene, Ind; Dibenz[ah]anthracene, D[ah]A and Benzo[ghi]perylene, B[ghi]P found in a non-washed moss subsample and in a subsample subjected to the cleaning procedure recommended in this work (n=3).

Graphical Abstract (for review)

