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Short Communication

Sample pretreatment to differentiate between bioconcentration and atmospheric deposition of polycyclic aromatic hydrocarbons in mosses

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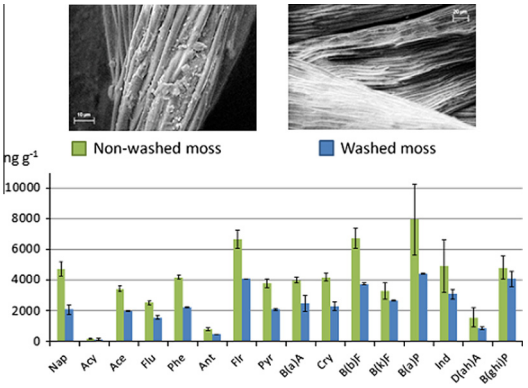
HIGHLIGHTS

- 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.

GRAPHICAL ABSTRACT



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ABSTRACT

In this first approach a 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) and submitted 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.

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) (Gerdol et al., 2002; Ötvös et al., 2004; Blasco et al., 2006). 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

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hydrocarbons (PAHs) as 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 pollutants in 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 (Sentenac and Grignon, 1981; Pérez-Llamazares et al., 2011; Spagnuolo et al., 2013).

Regarding the analytes to be studied in this work, PAHs are a large group of organic compounds with two or more 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 sample and (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 *B. 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 d^{-1}) and two highroads (N-634 and N-637 with 10 000 and 134 000 vehicles d^{-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 50 °C (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 magnifications up to 2000× 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 pÁ. Particles were observed as backscattered electron images and further subdivided in three size classes (<2.5 μm, 2.5–10 μm and >10 μm 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 °C, $5.4 \cdot 10^{-2}$ mbar; Telstar, Spain) before analysis. All solvents used were of HPLC grade and were obtained from

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	2× (15)
5	HNO_3 (10%) + ultrasonic bath	2× (15)
6	Milli-Q water + ultrasonic bath	15
7	HNO_3 (10%) + ultrasonic bath	15
8	Stream of N_2	5