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Distinguishing metal bioconcentration from particulate matter in moss tissue: Testing methods of removing particles attached to the moss surface

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HIGHLIGHTS

- The cleaning of surface particles on mosses is an unresolved issue
- The use of a nitrogen jet as cleaning procedure was ruled out
- The use of ultrasound treatments as cleaning procedure was also discarded
- Currently it is not possible to quantify the bioconcentrated metal fraction in mosses

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ABSTRACT

Accurate differentiation of the proportion of bioconcentrated metals (i.e. incorporated into cells) and the proportion that is not bioconcentrated (i.e. adsorbed at the surface) would lead to a better understanding of the uptake processes and would represent an advance in the use of mosses as biomonitors. Traditionally the methods used to remove contaminants that are not bioconcentrated were to wash the plant material with water or to apply the sequential elution technique, but nowadays both options are considered inaccurate for these purposes. The remaining possibilities are to clean the moss samples with a nitrogen jet or by power ultrasound. Samples of terrestrial moss *Pseudoscleropodium purum* (Hewd.) Fleisch. were collected from five sampling stations. Different nitrogen jet cleaning procedures and ultrasound cleaning procedures were applied to the mosses. To determine whether any of the treatments altered the membrane integrity of the moss samples, the concentrations of K were determined. The shoots were observed under a scanning electron microscope, and the size and number of particles were determined. Nitrogen jet cleaning was determined to be unacceptable because it damaged the phyllids and/or altered the membrane permeability and did not eliminate the particles from the moss surface. Moreover, ultrasound cleaning treatment should also discarded because of the loss of extracellular metals that are transferred to the water in which the moss is cleaned.

1. Introduction

The scientific literature includes numerous studies in which both native and transplanted terrestrial mosses have been used to biomonitor heavy metal contamination in the total tissue (e.g. Onianwa, 2001; Ares et al., 2012), as well as in the different fractions, as distinguished by sequential elution technique (see as example: Pérez-Llamazares et al., 2011a; Spagnuolo et al., 2011). In such studies, samples of moss are analysed to determine the concentrations of metals in their tissues, while avoiding any treatments that might alter these concentrations. However, it is still not clear exactly what these concentrations represent. The contaminants that are measured include those that are bioconcentrated (attached chemically to the cells) by the moss (and are present in both extracellular - elements bound to the cell wall and outer layer of the plasma membrane - and intracellular fractions - elements present within the cytoplasm or within cell organelles, as well as those bound to the internal layer of the plasma membrane; Brown (1995), as well as those that are attached physically to the surface of the moss but that do not interact with their tissues. The material that is not bioconcentrated (i.e. adsorbed at the surface) mainly comprises particles of different sizes that are deposited on the surfaces of the phyllids and caulids, although a small portion of such material may also be found in the intercellular spaces (elements in solution that bathe the exterior of the cell wall matrix and the cell membrane but that are not bound to the cells) (Brown, 1995).

The distribution of metals between the bioconcentrated and non bioconcentrated fractions is extremely variable and depends on the characteristics of the environment in which the moss grows (i.e. the amount and type of metal deposition, the form -soluble or insoluble- in

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which the metal is present, meteorological variables, etc.), and characteristics of moss species (e.g. endohydic or ectohydric). Furthermore, the dynamics of the contaminants differ in each of the fractions and are the result of independent processes that affect each location (i.e. the external surface and the intercellular, extracellular and intracellular locations). Furthermore, these locations do not show an integrated response to contamination (Aboal et al., 2010; Fernández et al., submitted for publication)). Clearly, the uptake of metals in moss is extremely complex and sometimes leads to results that are difficult to interpret.

Despite the above-mentioned problems, satisfactory results are often obtained by using terrestrial mosses to biomonitor heavy metals: to describe spatial and temporal patterns (see as example: Harmens et al., 2012), and to locate sources of contamination (Fernández et al., 2007). However, as disadvantages, sometimes the moss concentration is not related to the bulk deposition or lacks of relation between species concentration and shows for a single species a high temporal variability in short time lags studies (Aboal et al., 2010). Accurate differentiation of the proportion of bioconcentrated metals and the proportion that is not bioconcentrated would lead to a better understanding of the uptake process and would represent an advance in the use of mosses both in physiological/ecotoxicological investigations and for environmental monitoring. Indeed element uptake follows complex patterns influenced by various key factors (Tretiach et al., 2011).

One of the methods used to remove contaminants that are not bioconcentrated is to wash the plant material with water. However, this has been shown to be inefficient in regard to removing particulate matter from the surface of moss samples (Aboal et al., 2011). Another approach is the sequential elution technique (SET) (Brown and Wells, 1988), which enables separate quantification of the contaminants present in different cell locations. However, this technique has been shown to overestimate the extracellular location because of the solubilization of particulate matter attached to the moss surface (Pérez-Llamazares et al., 2011b).

After ruling out both of the above options, the remaining possibilities are to clean the moss samples with a nitrogen jet (Ducceschi et al., 1999) or by power ultrasound. Cleaning with a nitrogen jet appears suitable because the stream of nitrogen gas also dries the moss, and it has been shown that large quantities of particles are easily detached from dry moss (Fernández et al., 2010). Nevertheless, this method of cleaning is poorly described in the above-mentioned study (Ducceschi et al., 1999) (e.g. the N jet pressures and cleaning times are not indicated) and no attempt has been made to standardize the method. Power ultrasound is used to clean the surfaces of different materials during processing (e.g. to remove oxidation productions and fine coatings during processing of silica and heavy mineral sands: Farmer et al., 2000). In biomonitoring studies, ultrasound is used to clean particles attached to raptor feathers (e.g. Hudges et al., 1997; Castro et al., 2011; Debén et al., 2012). Nevertheless, as this method has not been used to clean moss it is not known whether it would be effective.

The present study aimed to determine whether there exist optimal combinations of i) pressure and time of application of a nitrogen jet and/or ii) intensity and time of application of power ultrasound, for removing particles from the surface of moss without affecting the membrane integrity of the samples.

2. Material and methods

2.1. Sampling

Samples of terrestrial moss *Pseudoscleropodium purum* (Hewd.) Fleisch. were collected from five sampling stations: i) an unpolluted site (X:537763; Y:4746262 ETRS89); ii) a steelworks (X:567565; Y:481633); iii) an aluminium smelter (X:622885; Y:4839099); iv) an Fe-Mn smelter (X:486127; Y:4754781); and v) an open cast quartzite mine (X:547070; Y:4737913). With the exception of the unpolluted site, all the sampling sites are located in environments characterised by high levels of particle deposition. Up to 2500 moss shoots were collected at each station. Before applying any treatment, the samples were acclimatized by exposure to a moisture-saturated atmosphere (Wells and Brown, 1990) for 7 days to ensure cellular membrane integrity (Pérez-Llamazares et al., submitted for publication).

2.2. Nitrogen jet cleaning procedures

Moss shoots were fixed with plastic clamps (10 cm long) for application of the N jet. The shoots were distributed so that 3 cm segments protruded from the clamp. The N jet was directed towards adaxial surfaces (an attempt was made to separate the phyllids from the caulids and to clean between them) and abaxial surfaces, and it was moved along the clamp during application. Five different N pressures (0.5, 1, 2, 4 and 8 bars) and five cleaning times (10, 20, 30, 60 and 120 s) were tested. Each of the 25 combinations of pressure and time of application was defined as an N jet treatment. Forty shoots were used for each treatment; in addition, 40 untreated shoots were used as controls. After treatment, the protruding sections of the shoots were cut from the clamp for analysis.

2.3. Ultrasound cleaning procedures

The ultrasonic cleaning procedure was carried out with ultrasound equipment of capacity 130 W (Vibra-cell VCX 130 PB, Sonics and Matherials Inc., USA). Segments (3 cm long) of shoots were submerged in 20 mL of bidistilled water. Five different ultrasound intensities (20, 40, 60, 80 and 100% of the capacity) and three cleaning times (10, 20 and 30 s) were tested in four sites for a total of 60 samples. The maximum cleaning time was established by taking into account that washing for more than 30 s may disrupt the equilibrium of extracellular cations, and thus some of the bioconcentrated material may be washed away (Sentenac and Grignon, 1981; Wells and Brown, 1990). Each of the 15 combinations of ultrasound intensity and cleaning time was defined as an ultrasonic cleaning treatment. Thirty-five shoots were used for each treatment; in addition, 35 untreated shoots were used as controls.

2.4. Chemical analysis

To determine whether any of the treatments altered the membrane integrity of the moss samples, three aliquotes of each sample were analysed as replicates. The first steps of the sequential elution technique (SET) developed by Brown and Wells (1988) and modified by Vázquez et al. (1999) were used. The experimental design did not include intercellular extraction, so that only the extracellular and intracellular fractions were studied. For the first compartment, shoots (approx. 0.1 g) were placed in 10 mL of 20 mM $NiCl_2$, with shaking, in two successive steps (45 + 30 min); the extraction medium was renewed between the two steps. For the intracellular location, the shoots were dried to constant weight (45 °C) to rupture the cell membranes, and the dry weight of the material was determined. The soluble intracellular metal was then dissolved by shaking for 30 min in 10 mL of 1 M HNO_3 acid. The concentrations of extra and intracellular K were determined by flame atomic emission spectrometry (Perkin Elmer 2100). Blanks were used to assess contamination during the extractions. All the determinations were above the quantification limit.

2.5. Microscopic analysis and image processing

For scanning electron microscopy analysis, small pieces (3–5 mm) of apical shoots were dried at room temperature, mounted on stubs with double-sided adhesive tape and coated with carbon. The shoots were observed (1000X) under an environmental scanning electron