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Methodological aspects of moss sample preparation. Effects of freezing and duration of washing on the cellular distribution of elements in *Fontinalis squamosa* Hedw.

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ABSTRACT

Aquatic bryophytes are widely used in biomonitoring studies, mainly because of their capacity to act as bioaccumulators. As different methods can be used to preserve and process moss samples, the results of elemental analyses may also vary, particularly if the contents of different cellular compartments are analyzed separately. In the present study, we compared the total concentrations of some elements and the concentrations of these in different cellular locations in frozen-thawed and fresh samples of the aquatic moss *Fontinalis squamosa* that were also washed for different lengths of time before analysis. We used the sequential elution technique (SET) to extract the different fractions, and we determined the concentrations of K, Mg, Ca, Cd, Co, Cu, Al and Zn in the extracts. The results varied depending on the element, cellular location and moss sampling site. In the moss samples processed after freezing, the greatest differences were in the intracellular concentrations of K, Mg and Cd, which were lower than in the fresh samples. Minor differences were found in the concentrations of elements in other cellular locations and in the total concentrations of elements. The increase in the duration of the initial washing step, carried out to remove soluble and particulate intercellular elements, may also cause the release of elements (e.g. K and Mg) bound to extracellular exchange sites. The concentrations in the other cellular fractions and the total concentrations were less affected by the washing duration. Neither freezing nor the use of long washing times are recommended for processing moss samples prior to extraction of elements by the SET. Further studies are needed to confirm and clarify the observations.

1. Introduction

Bryophytes are commonly used to biomonitor contamination in both terrestrial and aquatic environments (e.g. Mouvet, 1985; Fernández et al., 2000; Vázquez et al., 2013). Bryophyte species that have been used to monitor aquatic environments include the moss *Fontinalis squamosa* (Burton and Peterson, 1979; Gonçalves et al., 1994; Martínez-Abaigar et al., 2002; Zechmeister et al., 2003; Birk and Willby, 2010; Sossey-Alaoui and Rosillon, 2013), which is widely distributed throughout NW Spain (Vázquez et al., 2007).

Biomonitoring studies usually involve measurement of the total concentrations of individual contaminants in bryophyte tissues (Carballeira and López, 1997; Samecka-Cymermann and Kempers, 1998; Cesa et al., 2010). However, determination of the concentrations of contaminants accumulated in different cellular compartments yields more useful information about the bioavailability and toxic effects of the contaminants and a temporally more accurate picture of the contamination (Wells and Brown, 1995; Branquinho et al., 1999; Vázquez et al., 2000b; Figueira and Ribeiro, 2005; Pérez-Llamazares et al., 2011a; Spagnuolo et al., 2011; Fernández et al., 2013). The sequential elution technique (SET), first developed by Bates and Brown (1974) and subsequently modified by various authors (Brown and Buck, 1978a,b, 1979; Beckett and Brown, 1984; Branquinho and Brown, 1994; Vázquez et al., 1999; Pérez-Llamazares et al., 2009), enables separate and gradual extraction of the elements accumulated in different cellular locations. In the SET (Brown, 1995), the sample is first washed to remove the intercellular fraction, which consists of elements present on the outside of the cell and that are not bound to the cell. The next fraction extracted is the exchangeable extracellular fraction, which comprises metals that are bound to the cell wall and outer face of the plasma membrane. The subsequent step involves extraction of the intracellular fraction, which consists of the elements present in the cytoplasm in either a soluble or loosely bound

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form. The remaining fraction is the residual or particulate fraction of elements that are not extracted in the previous steps and that consist of insoluble elements present both inside and outside of the cells.

There is a risk that some of the elements that form part of a cellular fraction will be quantified in another fraction, because of the characteristics of the SET. Thus, for example, if the samples are washed for too long or energetically, exchangeable extracellular elements will be dislodged and included in the intercellular fraction. Thus, although long washing times were initially used (Bates and Brown, 1974; Brown and Buck, 1978b, 1979), the duration of the washing step was reduced to 30 s in later studies, to prevent alteration of the ionic equilibrium of the extracellular fraction (Wells and Brown, 1990; Vázquez et al., 2000a,b; Pérez-Llamazares et al., 2009). However, if the samples are not washed or the washing step is inefficient, part of the intercellular fraction may be included in the extracellular fraction. As far as we know, no previous studies have evaluated the effect of the duration of sample washing on the extraction of cellular fractions in aquatic mosses. Therefore, it is necessary to assess the effect of the duration of sample washing on the first cellular fractions extracted.

Another important aspect of the SET, and of biomonitoring studies in general, is the method used to preserve the samples between collection and analysis. It is not usually possible to process all of the material collected in extensive field surveys within a short time, and therefore some method must be used to protect the material from degradation. Drying and freezing are the most common methods used to preserve bryophyte samples (Samecka-Cymermann and Kempers, 1998; Fernández et al., 2000; Vázquez et al., 2007). However, drying has been found to alter the permeability of the cell membrane in moss, thus enabling the movement of mobile elements from the interior to the exterior of the cell, especially in species from aquatic environments (Brown and Buck, 1979; Brown and Brumelis, 1996). This method is therefore not suitable for differentiating elements accumulated in the different cellular compartments, at least in aquatic species. On the other hand, altered membrane permeability and release of mobile intracellular elements has also been observed in samples of the terrestrial moss *Pseudoescleropodium purum* that were frozen prior to application of the SET (Fernández et al., 2010). More information about the effects of freezing on the quantification of elements accumulated in the different cellular compartments is therefore needed, particularly in aquatic moss.

The aim of the present study was to investigate how freezing and the duration of the sample washing step affect the concentrations and cellular distribution of different metallic cations in the aquatic moss *F. squamosa*. The information obtained will improve our knowledge of the methods involved in the differential extraction of elements from samples of aquatic moss (and specifically of *F. squamosa*) and will also provide more information regarding quantification of total element contents. This will provide a basis for selecting the most appropriate methods to use in different cases.

2. Materials and methods

2.1. Sampling

Moss samples were collected at two sites (Mi-1, Mi-8) located in two small affluents of the river Miño (NW Spain). There are no important sources of contamination close to the sampling points.

Sampling site Mi-1 is situated in the river Anllo in a zone of slow moving current. At the time of sampling, the river was less than 1 m deep and 4 m wide. The river in this zone is shaded by a well-developed riverside woodland and is surrounded by grassland used for pasture. The substrate mainly comprises pebbles, and mosses and spermatophytes are both abundant at the sampling site. The moss samples were collected by removing them from stones on the riverbed or from tree roots.

Sampling site Mi-8 is located in the river Chamoso. At the time of sampling, the river was 0.5 m deep and 4 m wide. The current is of intermediate speed and the substrate is mainly formed of stones and gravel. Different species of moss coexist in the zone, which is shaded by the abundant tree vegetation growing along the riverside. There are some small vegetable plots in the surroundings of the area. The moss samples were collected by removing them from the surfaces of stones.

Samples of the moss species *F. squamosa* were collected at both sampling points. The material was rinsed several times in the river water to remove any large sediment particles and attached invertebrates; it was then squeezed gently to remove excess water before being placed in suitable containers. The samples were transferred to the laboratory in a portable refrigerator.

2.2. Sample processing. Treatments

In the laboratory, two subsamples of moss from each site were processed as follows: one was frozen at −20 °C for 7 days and the other was maintained at 4 °C until processing, always within 24 h of collection.

The fresh samples were processed directly, and the frozen samples were thawed at room temperature for 12 h before processing. To standardize the material used, apical sections (approximately 2 cm) were separated from the moss immediately before it was washed (Wehr et al., 1983; Satake et al., 1989). Groups of 60 apical sections (approx. 0.36 g fw) were then subjected to one of the following different treatments: (a) washing for 30 s; (b) washing for 6 min; (c) washing for 30 min (10 min + 20 min); (d) no washing. Each group of shoots were washed in 1 L of distilled water with shaking (with an orbital shaker).

2.3. Element extraction: sequential elution technique

After the apical sections were washed, they were removed from the water and placed (in groups of 20) in vials for extraction of elements. The elements were extracted from the different cellular compartments of the moss by using the SET, as outlined by Vázquez et al. (1999). To extract the extracellular elements, the samples were maintained for 45 min + 30 min in 10 ml of 50 mM $Pb(NO_3)_2$ as extractant (Merck, analytical grade), with shaking. Use of $Pb(NO_3)_2$ has proved efficient for extracting metal cations with medium to high affinity for extracellular binding sites, without altering membrane permeability (Vázquez et al., 1999). To obtain the intracellular fraction, the apical segments were dried at 60 °C for 24 h, weighed, and then further eluted in 10 ml of 1 M HNO_3 (Merck, Pure grade) for 30 min with shaking. To obtain the residual fraction, the remaining sample was digested with 6 ml of concentrated HNO_3 (65%) on a hot plate. All treatments were carried out in triplicate, with groups of 20 apical segments (approx. 0.04 g dw each group).

2.4. Analytical procedures

The concentrations of K, Ca, Mg, Al, Cd, Co, Cu and Zn were determined in the extracts obtained. The elements were determined by flame absorption spectroscopy (Perkin Elmer 2100), except for K, which was determined by flame emission spectroscopy (Perkin Elmer 2100), and Cd, which was determined by graphite furnace atomic absorption spectroscopy (Perkin Elmer A 600).

Analytical blanks were included to control the procedure. The final concentrations were corrected by subtracting the median concentrations of the blanks. The limit of quantification was