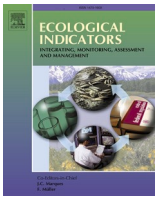
Ecological Indicators 131 (2021) 108192

Contents lists available at ScienceDirect

Ecological Indicators

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



Original Articles

An efficient method to wash out the particulate matter trapped by aquatic mosses

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ARTICLE INFO

Keywords:
Biomonitoring
Trace elements
Cleaning method
Fontinalis antipyretica
Pollution
Rivers

ABSTRACT

Aquatic macrophytes capture trace elements from water and store them in their tissues, which make them good biomonitors for these substances. But they also capture suspended particulate matter that could bias the concentration measurements in the moss tissues. To avoid this problem, many biomonitoring studies using mosses include a washing step in the sample preparation procedure.

However, no data is available on the quantities and variability of the particulate matter trapped by the moss, and the washing procedure has not been standardized between studies (washing time varied between 10 s to 30 min, divided in up to 7 washing steps). To study this, we extracted the particulate matter from samples of the moss *Fontinalis antipyretica* Hedw. collected in three rivers in NW Spain, washed them for 1, 2 and 5 min and collected the particulate matter extracted in each step. We also measured the contents of Al, As, Ba, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb and Zn in the tissues of the washed and unwashed mosses. We made a second experiment washing the samples only for 15 + 45, 30 + 30 and 45 + 15 s to determine if shorter washing times were adequate for cleaning the moss. In a third experiment we estimated the contents of particulate matter in moss samples collected monthly over a two-year period in two rivers in the same area.

The results showed that a) particulate matter can be as high as 40\% of the total weight of the sample, and that there were large variations within and between sampling sites, and over time. These results showed the need of washing the samples prior to their analysis; b) that two washings are needed for the removal of most of the particulate matter, but that 30 + 30 s are long enough to clean the moss, and c) that a 20:1 (or larger) water:moss relation is recommended to avoid saturation of the washing water, which would reduce the efficiency of the procedure.

We did not find significant losses of trace elements along the washing process, except for Cr in the three sampling sites, and Al, Fe and Pb in some of them. 1 min total washing time seemed the correct option both from the point of view of reducing the handling time of the samples and to prevent possible element losses.

1. Introduction

Aquatic macrophytes, particularly mosses, are good biomonitors because they capture the bioavailable fraction of pollutants from the surrounding water and store them in their tissues (Say and Whitton, 1983; Fernández et al., 2006; Vázquez et al., 2007). However, the moss mats also trap particulate matter suspended in the water, sometimes in such large quantities that Smith (1978) and Shacklette (1984) proposed to use them as particulate matter samplers for geochemical exploration studies. However, most biomonitoring studies do not determine the quantity of particulate matter trapped, nor its pollutant concentration. Therefore, the pollutant concentrations observed in the moss samples cannot be regarded as an unbiased measure of pollutant availability. Moreover, the quantity of particulate matter trapped in the samples can vary spatially and temporally, and independently of the availability of the pollutants in the water. An added problem is that the capture of particulate matter probably depends on the morphology of the species, as the particulate matter accumulates in the spaces between leaves and stems. These characteristics differ between species and to a lesser extent, within species. Therefore, we focused this study on the moss *Fontinalis antipyretica* Hedw., the most frequently used species in inland water biomonitoring studies (Debén et al., 2015). Despite the potential trouble

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https://doi.org/10.1016/j.ecolind.2021.108192
Received 16 June 2021; Received in revised form 6 September 2021; Accepted 7 September 2021
Available online 15 September 2021

that particulate matter can introduce in biomonitoring studies, we are not aware of studies measuring the quantities trapped by the moss and their variability. Using an indirect approach to this problem in a study on the terrestrial moss *Pleurozium schreiberi* (Brid.) Mitt., Dołęgowska (2017) and Dołęgowska and Migaszewski (2019) divided the statistical variability of the element concentrations between sampling period, sample preparation (washing or shaking) and within-site variability. Uncertainty due to sample preparation varied from 3.6 to 11.2%, but depended on the element and sampling period. We do not know similar studies on aquatic mosses, however.

Despite of this lack of information, many authors considered this as a relevant problem and applied the most obvious remedy: to wash the moss samples. In a review of the methods employed in 73 studies using native aquatic bryophytes as biomonitors, Debén et al. (2015) found that 64% of the studies washed the moss samples with water before the analysis to remove the particulate matter. This solution, however, poses two problems.

First, if the washing water and the moss do not come from the same place, the equilibrium of concentrations between them might change during washing. However, using local water to wash the samples complicates the design of the study, particularly in large-scale ones, and increases the workload. Therefore, the use of distilled or deionised water is much more convenient and common (49% of the studies reviewed in Debén et al. (2015) used them). However, they can act as extractants for the pollutants because the pollutant concentrations, ionic strength, etc, in the distilled water are lower than those in the river water. It has been observed that washing the moss with distilled water can alter the distribution of the chemical elements in different cellular compartments (as estimated by the sequential elution technique), i.e. releasing K and Mg from the extracellular exchange sites (Vázquez et al., 2015).

Studies with terrestrial mosses have also addressed this problem and considered it a potential bias source (Aboal et al., 2011). The situation is different because dry deposition is an important source of particulate matter for terrestrial mosses (Fernández et al., 2010; Spagnuolo et al., 2013). Therefore, washing them could release pollutants fixed to the surface of the tissues or dissolve those fixed to the particulate matter, which could be then recaptured by the moss tissue. These problems seem to be more serious in terrestrial studies because the conditions experienced by the moss while being washed (submersion, agitation) differ strongly from those in their environment, but they are not so different for aquatic mosses.

The second problem is that the washing procedure has not been standardised. Debén et al. (2015) showed that in the studies that explained the procedure in some detail (64%), total washing time varied from 10 s to 30 min, divided in up to 7 washing steps (1 or 2 were most common). Long, intense washings would remove more particulate matter, but transference of pollutants from the moss to the water would be more probable. Only two of the reviewed studies investigated how to wash the samples. Wehr et al. (1983) found that manual shaking was more adequate than more intensive, automated methods because it produced less damage to the material and needed less water. Lenarčič and Pirc (1987) proposed manual shaking of the moss samples in a plastic bag as a quick cleaning method. Similar studies with terrestrial mosses and trace elements showed that intensive methods (nitrogen jet, ultrasound cleaning) did not clean the moss completely and caused visible damage to the samples or element leaching (Spagnuolo et al., 2013).

With this state of affairs in mind, the objectives of this work were: a) to collect quantitative data about the amount of particulate matter trapped by *F. antipyretica* and their local (within a section of a river), regional (in different rivers of our region) and temporal variability; b) to determine whether washing produces appreciable losses of pollutants from the tissues, particularly trace elements, and c) based on this information, to determine the best procedure to wash the moss, i.e. the one that maximises the amount of particulate matter removed, minimising the washing time and simplifying the sample preparation 4work as much as possible.

2. Material and methods

The results come from three experiments that share part of the methods. The most complex will be described first, and later the particularities of the other two.

2.1. First experiment

With this experiment we investigated a) how the amount of particulate matter extracted from moss samples does depend on accumulated washing time, b) the variability between and within localities in the contents of particulate matter, and c) whether washing significantly changes the concentration of trace elements in the samples.

2.1.1. Sampling stations and sample collection

The sampling stations (for all experiments) were in the NW Iberian Peninsula (see Fig. 1). This is an area of mild and humid climate. Precipitations are low in summer and early autumn, a particularity of Mediterranean climates, but the yearly accumulated precipitations are larger than in southerner areas of the Iberian Peninsula. As a result, there are marked seasonal differences in water flow and level in the rivers of the area, but the overall abundance of precipitations allows for a dense hydrographic network. The samples were collected as follows (Fig. 1):

SS1 was in the Madalena river (43° 17′ 13.00′’ N, 7° 41′ 17.00 W (WGS84); altitude: 425 m.a.s.l.), downstream from the town of Vilalba, and nearly 1 km downstream from its sewage treatment plant. The moss was growing on the riverbed at this site. The current velocity varied throughout the sampling station.

SS2 was in the Ladra river (43° 9′ 48.30′’ N, 7° 43′ 5.80′’ W; 395 m.a.s.l). The mosses were collected up and downstream from a partly collapsed weir. The river is approximately 15 m wide in this area and the current velocity was slower upstream and faster downstream from the weir.

SS3 was in the Tinto river (42° 48′ 56.6′’ ’ N, 8° 37′ 37.30′’ W), a small river, 5 m wide at the sampling site. The river was a succession of pools and riffles. It was 1 km downstream from a wastewater treatment facility, but the immediate surroundings were cultivated areas and forest

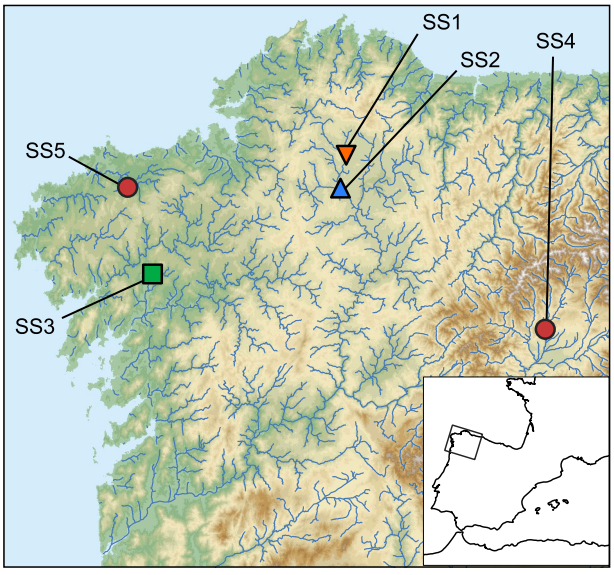

Fig. 1. Location of sampling sites in the study area. The symbols for the SSs will be used in the following graphs.

plantations.

The SSs were selected because they represented the diverse river typologies of this area, were easily accessible and the moss was abundant. Despite the presence of some pollution sources upstream, the river stretches did not show any visible symptoms of being polluted.

Each sampling station comprised a stretch of river about 100 m long, divided into three sections to collect a replicate in each of them to assess the within station variability. The distribution of the moss on the riverbed was always patchy, as it grew on tree roots, boulders or exposed bedrock, but not in areas covered with sediment. Therefore, the replicates were composed of material collected from at least ten fully submerged moss clumps. The moss was shaken in the river water to remove loosely attached sediment, debris and invertebrates, and gently squeezed to remove excess water. Each sample was introduced in a closed plastic bag, transported to the laboratory in refrigerated containers and stored in a refrigerator (6 °C). They were processed within 24 h of collection.

2.1.2. Sample preparation

In the laboratory, four sub-samples of approximately 7 g (wet weight) of 3 cm-long apical shoot segments were separated from each moss sample. This quantity was enough to produce at least 1 g of dry moss tissue, which was needed for posterior analysis. Only main shoots were selected, i.e. branches were discarded, to homogenise the age and morphology of the selected tissue. Damaged tissues or broken shoots without tips were discarded. Three sub-samples were subjected to different washing times: a) 1 min, b) 1 + 1 min, c) 1 + 1 + 3 min. analysis and left unwashed. The fourth was the control for the chemical The washing was done by placing the segments in 250 mL wide-mouth plastic vessels containing 100 mL of distilled water and shaking them by hand for the corresponding time. After each washing, the water containing the detached particulate matter was poured in centrifuge tubes, and the mosses left in the vessels. Another 100 mL of water were added to repeat the washing until completion of the series.

2.1.3. Determination of particulate matter contents

To determine the particulate matter extracted in each washing step, the water was centrifuged (Centronic BL, Selecta, Barcelona, Spain) at 3000 r. p. m for 3 min with 1 min start and stop ramps. The particulate matter adhered to the bottom of the tube, which allowed to pour out most of the water. It was resuspended and transferred to previously weighted glass vials using as little distilled water as possible (<30 mL). The suspension was allowed to settle for two days and most of the excess water retired by pipetting. Finally, the samples were left to dry in an oven at 50 °C for two more days and weighted.

2.1.4. Determination of trace element contents

After the washings were done, the moss shoots were partially dried by squeezing them between filter paper sheets. They were placed in a pre-weighted paper bag and weighted, dried at 50 °C for 48 h, and weighted again. Finally, they were ground in a tangential mill (MM400, Retsch, Haan, Germany), with zirconium oxide grinding containers and balls, and stored until analysis.

For the element determinations, aliquots of approximately 0.300 g of each ground sub-sample of moss were digested with a mixture of 8 mL of HNO_3 (69%) and 2 mL of H_2O_2 (33%) in a microwave oven (Ethos1 Plus, Milestone, Sorisole, Italy) for 15 min at 190 °C and 1000 W. Distilled water (Milli-Q, Millipore, Bedford, MA, USA) was then added to produce 50 mL of extract. The concentrations of Al, As, Ba, Cd, Cr, Cu, Hg, Fe, Mn, Ni, Pb and Zn in the extracts were then determined by Inductively Coupled Plasm-Mass Spectrometry (ICP-MS, mod. 7700x, Agilent, Santa Clara, CA, USA).

Four samples of standard reference material M2, *Pleurozium schreberi* (Steinnes et al., 1997), were analysed with the samples to control the quality of the extraction procedure. The percentage recoveries varied depending on the element (Table 1). The digestion procedure does not warrant the complete destruction of the mineral fraction in the samples, and this could cause the low recovery of Cr. However, the measurements were reproducible, showing low standard deviations, comparable to the certified and recommended values of the standard deviations.

Table 1
Measured, certified and recommended values (in italics) of four samples of M2 reference material. All values in μg g^{-1}.

	Measured			Certified	
Element	Mean	SD	% Recov.	Mean	SD
Al	201,3	41,4	119	*169*	*10*
Cr	0,24	0,03	35	0,67	0,19
Mn	593	51	111	*535*	*30*
Fe	152	17	110	138	12
Ni	0,69	0,06	73	0,95	0,08
Cu	2,78	0,23	74	3,76	0,23
Zn	23,3	1,5	92	25,4	1,1
As	0,112	0,025	107	0,105	0,007
Cd	0,086	0,004	81	0,106	0,005
Ba	12,4	0,6	90	13,7	0,6
Hg	0,044	0,005	126	0,035	0,004
Pb	2,63	0,17	79	3,33	0,25

2.1.5. Statistical analysis

Standard one-way ANOVA was used to test for the existence of significant effects of the washing treatments on the trace element content in the mosses. Due to the small number of replicates per treatment, we did not test the data for homoscedasticity or normality. Instead, a set of randomized ANOVA tests (Manly, 1997) were done on the same data and the results of the two analysis were compared. The randomized analyses were done in the following way.

The data were randomly assigned to the treatments, and the ANOVA was repeated to get an F-value. A distribution for the F-value under complete randomization of the data was obtained after repeating the randomization 9999 times. The probability of observing an F-value larger than the original one was estimated by comparing the value of the F obtained for the data with the distribution of the randomized values. To do the ANOVA analysis and to get the F-values we employed the function lm() in the R base package (R Core Team, 2020), and a custom R script to randomize the data, sort the F-values and estimate the probability value for the randomized tests. Exponential models were fitted to the observed relationships between accumulated extraction time and accumulated particulate matter (expressed as percentage of the moss weight), for the sub-samples washed 1 + 1 + 3 min. Function nls in R (least-squares fitting of non-linear models) was employed for these calculations. The equation had the general form:

$$p(t) = P\left(1 - e^{-bt}\right), \quad (1)$$

being $p(t)$ the accumulated proportion of particulate matter extracted at time t, P the total particulate matter proportion in the sub-sample, and b the slope of the curve at the origin.

2.2. Second experiment

The second experiment was designed to investigate whether shorter washing steps could be employed without losing cleaning efficiency. We collected a single moss sample in SS3 (in the central section) to use a material as homogeneous as possible. The strong flow conditions in the rivers at the time only allowed the collection of a limited quantity of moss, therefore we separated apical 5 cm segments to make twelve 5 g (fresh weight) sub-samples. They were washed with 70 mL of water to maintain the proportion moss:water used in the first experiment. Three sub-samples were washed for 1 + 1 + 3 min ("long washings") to obtain data to compare with the first experiment; three were washed for 15 + 45 s, three for 30 + 30 s and three for 15 + 45 s ("short washings"). The weight of particulate matter extracted was determined as in the first

experiment. Element concentrations were not determined in this experiment.

The exponential model (Eq. (1)) was fitted to the data of the long washings as in the first experiment.

2.3. Third experiment

The intent of this experiment was to obtain data of the variation in particulate matter content in the moss over time. It must be noted that this experiment was designed for another study, a two-year study intended to measure the changes in trace element content of mosses growing in two SS. This explains the different methodology used to determine the particulate matter content (see below). However, we considered that the data were relevant for the objectives of this study, as they allowed us to follow the within and between SS variations in particulate matter content over those years, and decided to include them.

The samples were collected (see Fig. 1) in the following SSs:

SS4 was located in the Cúa river (42° 38′ 5.00′’ N, 6° 43′ 54.15′’ W; 610 m.a.s.l). In this case, the moss was growing in an irrigation channel that collects water from the river 250 m upstream of the study location. Aquatic vegetation was growing abundantly throughout the channel.

SS5 was collected in the Calvar river (43°, 12′, 6.00′’ N, 8° 47′ 28.30′’ W). This river stretch was 5–8 m wide, with variable current velocities as it was a series of riffles and pools. The banks were devoted to corn cultivation or planted forests.

Three replicate samples were collected monthly in each SS following the same procedure as in the first experiment, but in this case only one sub-sample was prepared from each replicate by separating 15 g (fresh weight) of 5 cm-long apical segments (including small branches). The material was washed in 200 mL of distilled water for 1 + 1 min. After washing, it was partially dried by gently squeezing it between filter paper sheets, weighted and oven dried as before. Because the fresh moss was weighted before and after being washed, we were able to estimate the particulate matter as the difference in weights. It was a coarse estimate, influenced by the humidity of the samples (and the loss of a small quantity of leaves, probably loose before washing), but precise enough to show large temporal differences in particulate matter content in both SS (more on this later).

Mean daily fow data for the Anllóns and Cúa rivers were downloaded from the web pages of the respective water authorities (Augas de Galicia and Confederación Hidrográfica Miño Sil, respectively). The Cúa river gauging station was just besides SS4, but for SS5 it was in the main river of the basin to which the Calvar River belongs.

3. Results

3.1. Particulate matter extraction

Fig. 2A shows the results of the extractions of particulate matter in the first experiment, expressed as proportions of particulate matter (part. matter weight/moss weight). There were appreciable differences between SSs in the 1-minute washing results. The mean values and ranges of the proportions of SS1-SS3 were 0.067 (0.036, 0.099), 0.031 (0.012, 0.073) and 0.023 (0.019, 0.033), respectively. Therefore, there was large variability within and between SSs, that increased as the mean value of the SS increased. The following washings extracted lower proportions of particulate matter and the variability within and between SSs also diminished.

Fig. 2B shows the results for the sub-samples that were washed three times, but expressed as accumulated values. The exponential model fitted to each data series is also represented in order to make clearer the pattern of extraction of particulate matter over time and to facilitate the comparison between data series. The final accumulated value varied for each series, depending on the load of particulate matter in that sub-sample. Despite this, the extraction pattern was similar for all the series, with most of the particulate matter being extracted in the two first

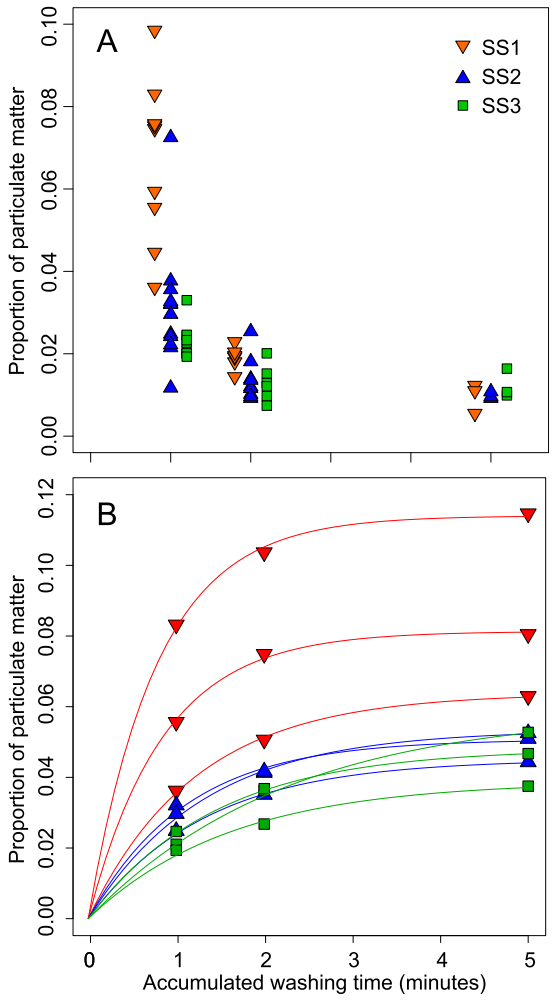

Fig. 2. Proportions of particulate matter extracted in experiment 1 (p. m. weight/moss weight). A) Particulate matter extracted in each washing step (not accumulated. all subsamples). The positions of SS1 and SS3 were displaced along the X axis to make the graph clearer. B) Results of the extraction of particulate matter as accumulated proportions. Only the subsamples that were washed three times are represented. The lines show the exponential models that were fitted to the data series.

minutes, and the curves arriving to their asymptotic part between two and five minutes.

Fig. 3 shows the results of the second experiment. Graph A shows the particulate matter extracted in the long washings and the models fitted to each series. The model fitted to them was the same as in experiment 1 (Eq. (1)), which showed that the washing process was similar in both experiments. The moss contained more particulate matter in this experiment than in experiment 1, so the final accumulated proportions were larger. The differences between the series were small, because we collected a single sample to reduce them as much as possible. The flattening of the curves was not so pronounced as in experiment 1, i.e. the washing was less effective.

Graph B shows the results of the short washings as cumulative values. The proportions of particulate matter extracted during the first 30 and 45 s washings were similar for all sub-samples, and slightly larger than those extracted in 1 min washings in the long-washing series (graph A). The second washings of these sub-samples (30 and 15 s) also extracted similar proportions from each sub-sample, and the accumulated proportions were like those extracted after 5 min in the long-time washing.

The results for the 15 + 45 s washing were less consistent. The proportion of particulate matter extracted from one sub-sample was like

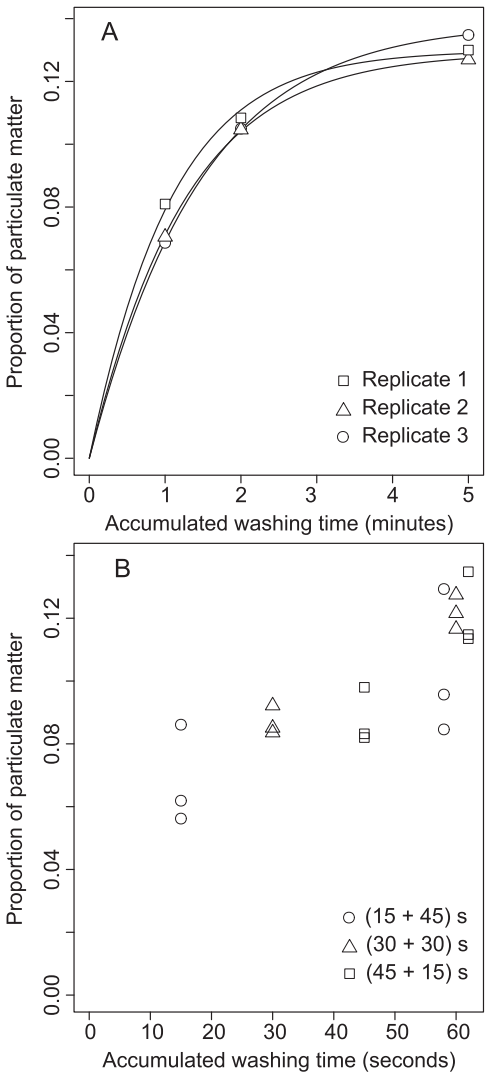

Fig. 3. Results of experiment 2. A) Accumulated proportion of particulate matter extracted in the long time washings as function of accumulated washing time. The lines show the models adjusted to the data. B) The results for the short time washings. The data for 60 ~ s washing were displaced along the X-axis to avoid cluttering.

those extracted in the other series, but for the other two were lower.

Fig. 4 shows the results of the third experiment. It contains the proportions of particulate matter in the samples collected in SS4 and SS5 and, to add some hydrological background, the mean daily flow for the Cúa and Anllóns rivers. The proportions of particulate matter were quite variable, both within the same collection data and between collection dates, but proportions as high as 30–40% were observed at both sites on some occasions. The variability showed by the data agrees with what we observed in the laboratory when cleaning these mosses. The turbidity of the water after the washing was very different between months and replicates, sometimes strikingly so. Equally variable was the proportion of coarse particulate matter, which settled quickly in the beaker where the water was collected.

3.2. Trace element contents

Despite the existence of some sources of pollution upstream of SS1 and SS2 the element contents were relatively low for all the measured elements. A comparison of the means of the unwashed samples with the means reported in Vázquez et al. (2007) (for a set of 74 samples of *F*

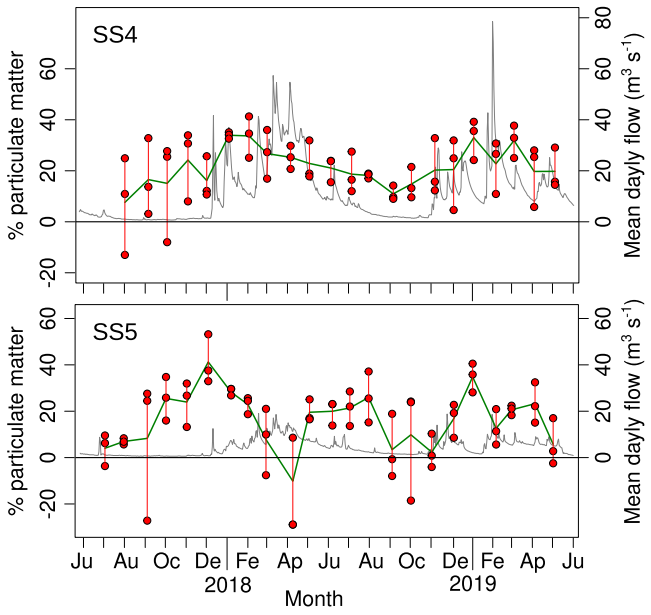

Fig. 4. Estimates of particulate matter content in the samples of the third experiment (red dots). The green line joins the mean values of the monthly samplings. Daily mean flow data in the rivers Cúa (SS4) and Anllóns (SS5) were also included to show the hydrological context.

antipyretica collected in a survey of the area represented in Fig. 1) showed that most of the means of our samples were in the interval mean − 1 s.d of the values reported there. Only Al, Cr and Ni were even lower.

Fig. 5 shows the trace element contents measured in the samples of experiment 1. The stars in the graphs denote the significance level of the f-test in the ANOVA between washing times. Most of the analysis did not show significant differences, which indicated that the washing did not change the element contents in the samples. However, many elements in SS1 and some in SS3 showed an increase of concentrations when washed 1 and 1 + 1 min followed by a decrease in the last washing. Cr was the unique element that consistently showed significant concentration losses along the washing process. Pb also showed similar losses but only in SS1 and SS2. Even for these two elements, the losses after 1 min samples were small.

4. Discussion

4.1. Particulate matter content

The results of experiments 1 and 2 showed that the contents of particulate material can be very variable, ranging from <2% to 15% of the dry weight of the samples (Figs. 2 and 3). Watershed characteristics (bedrock, climate, soils, soil cover and use) were different at each SS, as well as the particular characteristics of the river (order, width, slope, flow) and all of them can influence the particulate load in the water and therefore, the quantity trapped by the moss.

The variability within SS was also large. The samples comprised a number of moss clumps, and branches were randomly selected from them to create composite sub-samples. The growing conditions of each clump were different regarding water velocity, which varied in short distances. Despite the averaging effect of the composite sampling, the differences between sub-samples within the same SS were appreciable. They were larger in the sample with the largest load, but occasional replicates with large particulate contents appeared in SS2 (Fig. 2).

It must be noted that the morphology of *Fontinalis antipyretica* is very variable (Welch, 1960). Part of this variability is an adaptation to the prevalent flow velocity in the surroundings of the moss (Biehle et al., 1998). In fast-flowing spots it develops smaller leaves and tougher and

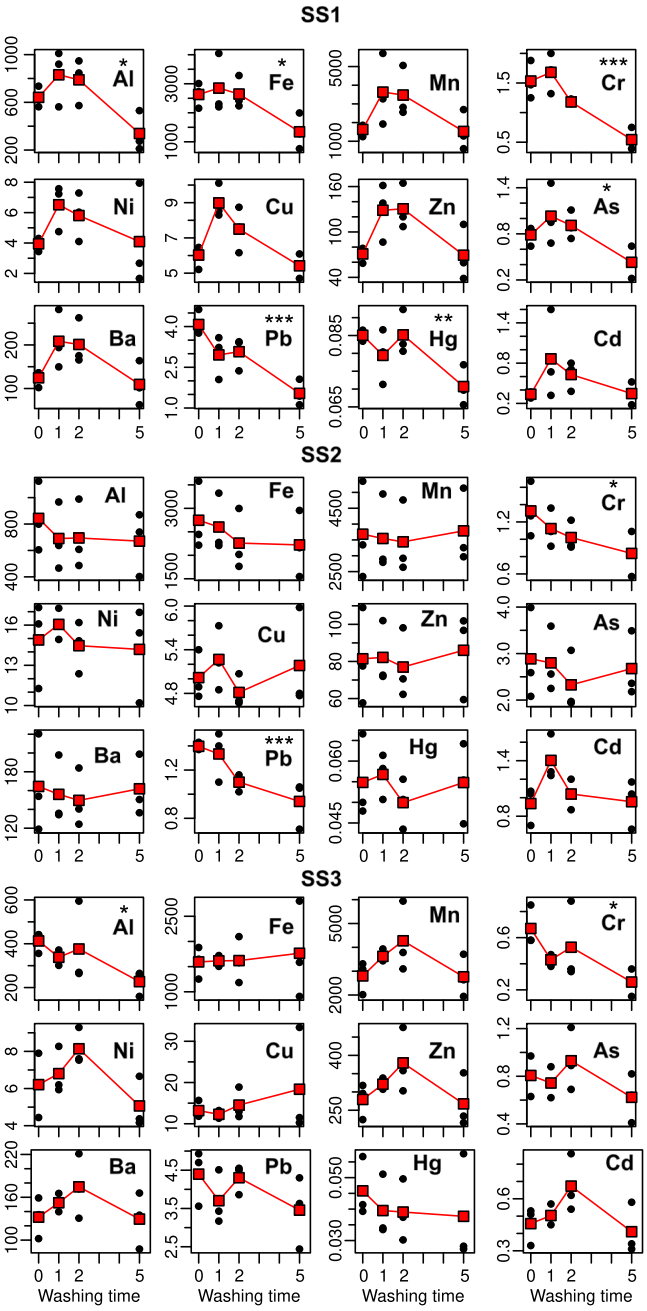

Fig. 5. Metal contents (μg g^{-1}) in unwashed samples (time = 0), samples washed for 1 min, 1 + 1 min and 1 + 1 + 3 min. Red squares are the mean values (n = 3). Results of the ANOVA f-tests: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

shorter stems than in calmer waters. We observed such variations in SS2 and SS3, for example. As the moss morphology changes, so does its capability to trap particulate matter.

The results of experiment 3 added a temporal perspective. The proportions of particulate matter changed irregularly from month to month and values as high as 40% were observed on some occasions. The flow conditions in SS4, an artificial channel, were more stable over time than in SS5, a small stream. Despite this, the particulate load in the mosses from both places showed large differences between months. Note that these data underestimated the quantity of particulate matter because the moss was drying while the segments were being collected, and were wetter after washing the samples. Sometimes the squeezing after the washing was not as thorough as it should be (we did not consider it a very relevant step in the procedure at that time), which caused the negative values in the data set (Fig. 4). Although the quantitative accuracy of the results of the experiment 3 could be questioned, they agreed to our own qualitative observations during the laboratory work, i.e. that there were large variability between and within samples, and that large contents of particulate matter are not rare events. They were also in accord with the observations commented before (Smith, 1978).

These results showed the potential of the particulate matter content to act as a biasing factor in biomonitoring studies. If its pollutant loads were large in relation to those in the moss tissues, the bioavailable pollutants in the water would be overestimated. But in the opposite case they would be underestimated, as the particulate matter would contribute mass but not pollutants. The bias may vary within and between SSs and also temporally, in unpredictable ways. It can also vary on a per-pollutant basis. These biases could hinder the interpretation of the

results. In regional-scale surveys, for example, which usually include many SSs, each with different flow regime and particulate and pollutant loads, this could be a serious problem. Therefore, washing the samples is necessary to remove part of the noise and bias in the data and to improve the comparability between samples.

4.2. Trace element contents

A potential problem caused by the washing is the loss of elements from the moss tissue. The results of the ANOVA for the element contents in the washed and unwashed samples were not significant in most cases (Fig. 5). Cr consistently showed significant differences in all three SS, and Al, Fe and Pb only in some of them. In all the significant cases, the differences in mean concentration between the unwashed sub-samples and those washed for one minute was smaller than the differences after five minutes. This pattern cannot be attributed to the washing of the particulate matter and their associated elements, because in such case the loss of elements should be larger in the first washing, when most particulate matter is washed out. This suggests that element losses from the tissues could occur during long washings, at least for some elements, but losses during short washings are small and not significant, given the variability of the data.

The concentrations of the unwashed material of SS1 and SS3 (Mn, Ni, Zn, Ba, Cd) were lower than those in samples washed for 1 and 2 min. However, due to the dispersion shown by the data, the ANOVAs did not detect significant differences between the means. Therefore we considered these patterns as a random effect of the subsampling procedure.

4.3. Washing procedure

The last question is how to clean the samples, i.e. how many washings to do, how much time per washing is needed and how much water should be used. First, the results of the long washing series showed that the release of particulate matter proceeded consistently for all the samples. This was supported by the fact that the same equation could be adjusted to all the series in experiments 1 and 2 (Figs. 2 and 3). Note that we do not claim that the mathematical form of the model itself has some particular meaning, as we adjusted a continuous function to the results of a stepwise procedure. It is only a tool to visualise the consistency of the washing procedure. These results showed that designing an standardised procedure of general validity is attainable.

Second, short washing times were enough to retire most of the particulate matter. A minute of total washing time extracted comparable proportions of particulate matter as a 5 min series of washings. This is a good notice, given that such short times cause only small losses of elements from the moss. The results also showed that two washing steps are necessary to clean the samples. The results of the short washings in experiment 2 suggested that two washings of 30 s each would be enough to extract most of the particulate matter or, at least, to extract as much as long washings. The results for 15 + 45 s were less clear as the particulate matter content of two of the replicates was lower than in the other two washing series. This occurred in the two washings, which suggests that it was not caused by a too short first washing because in this case, the second washing would have extracted more particulate matter in comparison to what occurred in the other two series. In fact, the contrary was observed, and this suggests that those replicates contained less particulate matter than the third.

Using very short washing times is problematic, as well. It is difficult to wash for exactly 15, 30 or 45 s, as small delays can be produced when manipulating the samples (filling and emptying the vessels, etc.), but their relative effect on the real washing time becomes smaller as the washing time is longer. Based on our experience in the laboratory, we consider that 30 s per washing seems to be a suitable compromise between handling and washing time.

The results of experiment 2 also suggested that water saturation by the particulate matter could happen during the first washing, as the 30 and 45 s washings extracted the same quantity of particulate matter. The solution to this problem would be to use more water. We arbitrarily decided to use 100 mL for 7 g of moss and maintained this proportion in the following experiments, but we could have used 200 mL or any other volume. Using more water needs larger vessels and more space in the laboratory when handling many samples simultaneously. From a practical point of view, making two short washings with a 20:1 proportion between water and fresh moss weight is a suitable compromise. Our experiments used lower ratios and worked correctly, and using this ratio, 200 mL vessels are small and easy to handle, and would be adequate for samples up to 10 g of fresh moss. The exact volume could be varied depending on the quantity of moss per sample, and whether the samples are expected to be heavily loaded with particulate matter. In these cases, the water volume can be increased. Consider this recommended ratio as a minimum, not a fixed value.

The asymptotic character of the models adjusted to the long-washing data in experiments 1 and 2 indicate that complete cleaning of the samples is not attainable. This has been observed repeatedly in studies with other water macrophytes (Freitas et al., 1993), terrestrial mosses (Spagnuolo et al., 2013) and marine algae (Gledhill et al., 1998). But our results also showed that most of the particulate matter can be eliminated with short washings. If this were not considered enough, estimations of pollutant content in the particulate matter and of the amount of particulate matter could be used to correct the pollutant values measured in the samples (Luoma et al., 1982; Barreiro et al., 2002; Freitas et al., 1993).

5. Conclusions

The conclusions of this study were that washing the aquatic moss is a necessary step in the sample preparation prior to the analysis of trace elements. If this is not done, the uncertainty about the real composition of the samples would be large and the comparability of the samples not great. In order to achieve high efficiency in washing out the particulate matter, two successive washing steps, each of 30 s of agitation, must be applied, setting the water/moss wet weight proportion equal to or greater than 20:1.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Funding: This work was supported by Ministerio de Ciencia, Investigación y Universidades (Spain) project CTM2015-70578-P. The authors belong to the Galician Competitive Research Group GRC/GPC2016-002. These programs are co-funded by FEDER (EU). The sources of funding did not intervene in the preparation of the research or the paper.

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