Science of the Total Environment 932 (2024) 173045

Contents lists available at ScienceDirect

Science of the Total Environment

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

A method for studying the influence of Mn oxyhydroxides on the trace element content of aquatic bryophytes

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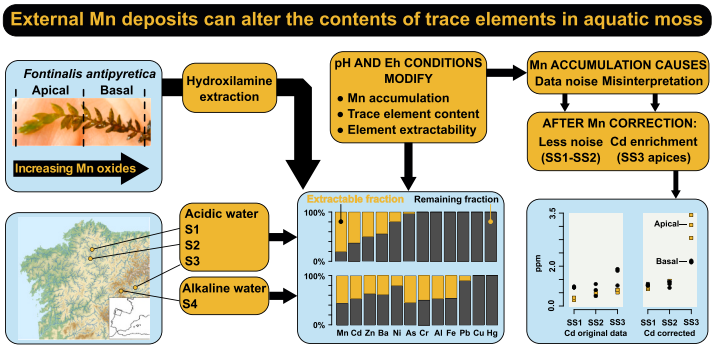
HIGHLIGHTS

- Mn oxohydroxides, enriched in Cd, Zn, Ba and Ni, accumulate on aquatic moss surfaces.
- Hydroxylamine extraction released up 84 % of Mn in the samples.
- Variable Mn accumulation modifies the trace element contents of the samples.
- Biomonitoring data might be misinterpreted if Mn contents are not considered.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Daniel Alessi

Keywords:
Bryophytes
River
Biomonitoring
Mn oxyhydroxides
Dihidroxylamine extraction
Gamma distribution

ABSTRACT

The main objective of this study was to develop and test a method of separating externally deposited Mn oxyhydroxides and co-precipitated elements from samples of aquatic moss (the moss *Fontinalis antipyretica*). The method, which uses 0.1 M hydroxylamine to dissolve the oxyhydroxides, was tested with samples collected in rivers with slightly acidic, well-oxygenated waters, where high rates of Mn precipitation occur. The method was effective (it extracted up to 84 % of the Mn) and selective (Fe oxyhydroxides were not extracted). The elements Ba, Cd, Zn and Ni were associated with the Mn oxyhydroxides, while Al, As, Cr, Cu, Fe, Hg and Pb were not. Deposition of Mn therefore increased the concentration of some elements in the moss samples. However, as Mn precipitation depends on Eh and pH, which are independent of the concentrations of the elements in water, the relationship between water and moss element concentrations is not clear (i.e. the data are noisy). This is a problem in biomonitoring studies, which assume a close relationship between element concentrations in moss and water. The value of the proposed extraction method is that it can be used to correct the effect of Mn deposition. We present an example of this correction applied to the Cd concentrations in the test data. We found that the noise introduced by the Mn, including age-related effects (observed by comparing concentrations in 0–2.5 and 2.2–5.0 cm sections from the shoot apex), can be reduced. Additionally, the correction revealed recent increases in Cd concentrations in one site that were not observed in the uncorrected data. Another finding of interest was the low content of total Mn and different extractability (of most elements) observed in moss samples

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https://doi.org/10.1016/j.scitotenv.2024.173045
Received 15 January 2024; Received in revised form 18 April 2024; Accepted 5 May 2024
Available online 9 May 2024

collected in alkaline waters. Finally, we discuss how future studies designed for different environmental scenarios can benefit from application of the proposed method.

1. Introduction

Aquatic bryophytes can be used to biomonitor trace elements in aquatic systems, fulfilling the criteria proposed by Phillips (1980). Trace elements are accumulated through complex processes that vary depending on their state in the water, i.e. whether they are dissolved or bound to solid particles (Brown, 1984; Brown and Wells, 1988; Mouvet and Claveri, 1999; Vázquez et al., 1999a). As a result of these interactions, the element content of bryophytes is divided between the interior of the cells and several external storage sites.

Dissolved elements can enter the cell by ion uptake across the plasma membrane via a combination of active and passive mechanisms (Tyler, 1990; Pickering and Puia, 1969). Once inside the cell, the elements can disrupt physiological processes such as photosynthesis (Brown and Wells, 1990), growth (Sidhu and Brown, 1996), growth and lipid metabolism (Guschina and Harwood, 2000). They can also be adsorbed by phosphodiester, carboxyl, phosphoryl, amine and polyphenol groups, which are abundant on the outer faces of the membrane and cell wall. These cell structures have a high cation exchange capacity. The adsorbed elements are not as toxic as the forms inside the cell, but they can be transferred to the cell at a later stage if they are redissolved.

Mineral and organic particles in water also contain trace elements. The trace elements can form part of the structure of the particles or they can be adsorbed on the particle surface. Moss clumps retain particles as water flows through them. The particles then become attached to the moss surface and accumulate within the crevices created by the plant structure. Accumulation of particles varies greatly depending on the suspended load in the water and other hydrological factors, but it can reach up to 40 % of the dry weight of the moss (Real et al., 2021; Zotina et al., 2023). Particulate matter can thus contribute significantly to the total trace element content of mosses.

Finally, Fe and Mn oxides can precipitate on the surface of mosses (Tipping et al., 2008; Vázquez et al., 2020; Vincent et al., 2001; Whitton et al., 1982). Accumulation may be great enough to generate dark oxide crusts on the older parts of the plants. Studies concerning soil and aquatic sediments have focused on Fe and Mn oxides because of the role of these compounds in regulating trace element concentrations (see the review by Tebo et al., 2004). However, they have generally been overlooked in biomonitoring studies, although they contain and can adsorb trace elements in high quantities (see below).

This study investigated the influence of Fe and Mn oxides on trace element accumulation in aquatic mosses. The processes that affect the formation of these compounds are summarized below.

1.1. Chemistry of Fe and Mn oxides

Aquatic systems contain Fe and Mn in the form of dissolved di- or trivalent cations, which are oxidized to hydroxides or oxides (hereafter we will refer to these collectively as oxides, for simplicity), depending on the Eh and pH conditions. As they are less soluble than the dissolved cations, the oxides are precipitated. These reactions can be represented in Eh-pH diagrams, which show the equilibrium lines for pairs of the chemical species considered. Fig. 1 shows the superimposed diagrams for Fe—C—O—H and Mn—C—S—O—H systems, highlighting the differences between these elements. To provide some context for the present study, we have included the typical pH and Eh ranges for the rivers in the study area (Antelo Cortizas and Arce Vázquez, 1996).

The positions of the equilibrium lines for Fe^{3+}, Fe^{2+}, and Mn^{2+} in Fig. 1 show that Mn and particularly Fe are more soluble in acidic waters. The equilibrium lines for Fe^{2+} and Mn^{2+} are roughly parallel but their relative positions show that Fe is oxidized at lower Eh and pH than Mn. Therefore, under near-neutral pH conditions, more Mn should be precipitated. The studies of Kimball et al. (2010) and Williams et al. (2015) are clear examples of this differential behavior in a natural system.

1.2. Biochemical processes

The Eh-pH diagram shows that elevated oxygen levels in the water (high Eh) favour oxidation of Fe and Mn. Physical processes (e.g. turbulence) and biological processes (e.g. photosynthesis) cause increases in oxygen concentrations. In addition, photosynthesis can increase the pH under certain conditions. As oxygen is released at the plant surface, the layer of water closest to the surface is the most affected. In addition, oxygen accumulation is enhanced when renovation of the water layer is limited by slow movement of water (e.g. inside moss clumps and areas of low or slow flow). Thus, the surface of plants provides favourable conditions for oxide formation and deposition. This is especially true for mosses, because the leaves of many species have only one cell layer, allowing the phothosynthetic cells to interact directly with water. X-ray microanalysis has revealed high concentrations of Mn in precipitates accumulated on the leaves of *Sphagnum auriculatum* and *Fontinalis antipyretica* (Sérgio et al., 1992, 2000). Similar precipitates have been

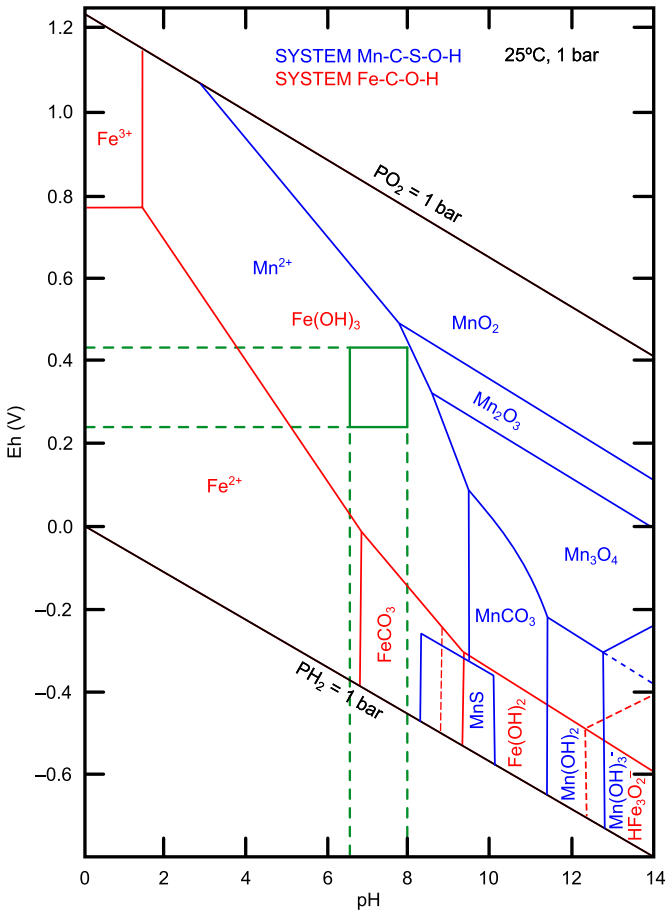

Fig. 1. Combined Eh–pH diagram for Fe (red lines) and Mn (blue lines). The rectangle shows the Eh and pH ranges in the rivers in the study area (Antelo Cortizas and Arce Vázquez, 1996).
Modified from Brookins (1988).

observed in other macrophytes (Oborn, 1963).

The periphyton also plays an important role in biological processes in mosses. The periphyton consists of microalgae, bacteria, fungi, protozoans and small metazoans that attach to the surface of aquatic plants (such as mosses), stream beds, boulders and other solid surfaces (Costerton et al., 1995). The layered film structure they create enhances the precipitation of several elements, particularly Mn, thus playing a significant role in stream biogeochemistry (Spiro et al., 2010; Jørgensen and Revsbech, 1983; Haak and Warren, 2007; Tebo et al., 2004; Miyata et al., 2007a, 2007b). Bacteria in particular, can enhance the oxidation of Mn by a 1:1000 factor compared to spontaneous reaction rates at neutral pH (Spiro et al., 2010). The freshly precipitated Mn oxides are autocatalytic and promote the oxidation of Mn(II). The oxidized Mn form three dimensional structures constituted by MnO_6 octaedra (e.g. todorokite) or staked sheets of edge-sharing octaedra (birnessite). The former are more abundant in marine nodules and the latter in soils and freshwaters (Spiro et al., 2010).

1.3. Mechanisms of cation capture

Layer type Mn oxides show many defects in the crystal structure (vacancy sites without Mn ion and weak crystallinity) (Spiro et al., 2010). Biogenic Mn oxides have more defects than their non-biological counterparts. The defects generate negative charges in the sheet surface, which are compensated by hydrated cations placed between the layers. However, the outer surface of the staked layers and the sheet borders have uncompensated charges, which behave as variable charges, i.e. pH-dependent charges. The point of zero charge of the oxides is low (e.g. pH = 1.75 for birnessite) (Feng et al., 2007), therefore at near-neutral pH the surface charge of the particles is negative. Since the particles of biogenic Mn oxides are very small their surface area is very high, resulting in a very high cation adsorption capacity (Spiro et al., 2010; Tebo et al., 2004). Zhou et al. (2015) found that the capacity of biogenic Mn oxides to adsorb Pb(II), Cd(II) and Zn(II) exceeded that of abiotic Mn oxides by 7–8 times (see also Miyata et al., 2007b).

Finally, some elements, notably Ni, can be incorporated into the crystal lattice by isomorphic substitution of the Mn ions.

1.4. The combined role of environment and time

Chemical and biochemical processes are influenced by the surrounding environmental conditions. The water in most rivers in the study area is well-oxygenated and mildly acidic to neutral, causing Mn to precipitate at a higher rate than Fe. Vázquez et al. (2020) observed 25-fold increases in Mn concentrations along the 5 cm apical segments of moss branches, while Fe increments were only eight-fold. Some trace elements underwent comparable increases, while others did not. For example, the concentrations of Cu or Hg remained stable along the shoot, while Ni showed a maximum 20-fold increase. The increasing concentrations suggest that precipitation of oxides is a continuous process that results in higher deposition on older tissues. However, these authors did not observe such increases in mosses growing in alkaline waters.

1.5. Working hypothesis and objectives of this study

The Mn oxides deposited on the surface of mosses may contain a large fraction of the total contents of at least some of the elements. Therefore, the processes governing Mn deposition can alter the concentrations of other elements, independently of variations in the pollutant content in the water. The effects of pollution and Mn accumulation on the elemental concentrations may therefore be intermingled, making the findings difficult to interpret. In addition, the affinity for Mn may vary depending on the element considered, adding a further layer of complexity. A method of measuring the oxyhydroxide concentrations is therefore needed in order to clarify how they influence element uptake.

No such method has been developed to date, because previous biomonitoring studies have overlooked the role of oxides in the accumulation of elements in mosses. The aim of the present study was to develop a method of separating the external Mn oxides and associated elements from moss samples. The procedure must be sufficiently selective so that it extracts Mn oxides, but not other oxides present in the sample, such as Fe oxides.

2. Material and methods

2.1. Sampling stations and sample collection

The aquatic moss *Fontinalis antipyretica* Hedw. was selected for this study. This species is common in the rivers in the study area and it is the most frequently used in aquatic biomonitoring studies (Debén et al., 2015).

The sampling stations were located in rivers within the NW Iberian Peninsula. The climate in the area is mild and humid. Precipitation is lower in summer and early autumn, as is characteristic of Mediterranean climates, although the yearly total exceeds that in southern areas in the Iberian Peninsula. The rivers in the area therefore undergo distinct seasonal fluctuations in water flow and level, but the abundant rainfall maintains a dense permanent hydrological network.

The samples were obtained from four different locations.

SS1 was located 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 almost 1 km downstream from the town's sewage treatment plant. Upstream, the territory is devoted to agriculture, livestock farming and forestry. The moss was growing on the riverbed at this site. The current velocity varied throughout the sampling station.

SS2 was located in the Ladra river (43° 9′ 48.30″ N, 7° 43′ 5.80″ W; 395 m a.s.l). The moss samples were collected upstream 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 of the weir. No specific source of pollution was identified in the vicinity of this SS.

SS3 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 which captures water from the river 250 m upstream from the study location. Aquatic vegetation was growing abundantly throughout the channel. An open-pit coal mine, which covers an area of 520 ha (currently active part), is present 21 km upstream of the SS.

SS4 was a karstic spring arising from a limestone outcrop (42° 35′ 23.63″ N, 7° 30′ 25.35″ W; 820 m a.s.l.). The moss was growing abundantly in a pool formed at the point where the water flows out of the rock. This is a mountainous area with a low population density, and there is no relevant source of trace element pollution in the surroundings of the SS.

At SS1–SS3, the pH of the water ranged between 6.5 and 7, falling within the typical range for rivers in this region (Antelo Cortizas and Arce Vázquez, 1996) (see Fig. 1). The water in SS4 is alkaline (pH 8.0) because the rock is calcareous. The combination of alkaline water and *F. antipyretica* is rare in the whole study area, so only one sample of this type was included in the study.

Each sampling station comprised a stretch of river about 100 m long, which was divided into three contiguous sections for collection of one replicate moss sample from each. Individual samples of *F. antipyretica* were randomly collected from 10 to 15 submerged clumps in each section. Moss grew on tree roots, boulders, and exposed bedrock within the riverbed, but not in areas covered with mobile sediment, resulting in a patchy distribution. In SS4, the moss covered a small area of only 50 m^2, and the three samples were collected at regular distances from the spring source. The moss samples were shaken in the river water to remove loosely attached sediment, debris and invertebrates. Excess water was then gently squeezed out. The samples were placed in plastic bags, which were then sealed, held in refrigerated containers during

transportation and stored at −20 °C in a freezer until processing.

2.2. Sample preparation

Prior to processing, the samples were allowed to thaw overnight in a refrigerator at 6 °C. Two subsamples each of approximately 5 g of fresh material were collected from each sample by removing the apical 5 cm from randomly selected shoots. To remove any particulate matter attached to the surface, each subsample was washed by placing it in a plastic bottle with 200 mL of distilled water, shaking for one minute, discarding the water and repeating the process one more time (Real et al., 2021). The washed apices of the first subsample were divided into two 2.5 cm segments (AS, apical segment; BS, basal segment), which were analysed to determine any differences in element content along the stem. The second subsample, used for Mn extraction (see below), was processed in the same way as the first, but without dividing the segments until the extraction was completed. Thus, each SS yielded 12 subsamples for element analysis (3 station sections × 2 extraction treatments × 2 shoot segments). Each subsample was placed in a pre-weighed paper bag and dried for 48 h at 50 °C, to prevent loss of volatile elements. Finally, the subsamples were ground in a ball mill in zirconium oxide containers (Mixer Mill MM400, Retsch, Haan, Germany) and stored at room temperature until analysis. Due to a shortage of material, only two replicates were available for SS4.

2.3. Determination of trace element contents

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 Plasma-Mass Spectrometry (ICP-MS, mod. 7700×, Agilent, Santa Clara, CA, USA).

Four samples of standard reference material M2, *Pleurozium schreberi* (Steinnes et al., 1997), were analysed along with the samples for quality control of the extraction procedure. The percentage recovery of the elements varied between 79 and 119 %. The measurements were reproducible, with low standard deviations, comparable to the certified and recommended values. Recovery of Cr was poor (35 %), probably because the digestion procedure does not guarantee complete destruction of the mineral fraction in the samples.

2.4. Mn extraction

We are not aware of any previous attempt to extract the Mn oxides deposited on moss surfaces. However, this is common practice in soil and sediment research, and diverse extraction solutions have been used (Chao, 1972; Tessier et al., 1979; Ryan and Gschwend, 1991; Real et al., 1994; Dong et al., 2001). Nonetheless, the methods used are not selective for Fe or Mn oxides and do not fully dissolve the oxides (amorphous oxides are easier to dissolve than more crystalline forms), and the extracted elements can potentially be reabsorbed by other chemical fractions of the samples (Tipping et al., 1985; Kheboian and Bauer, 1987; Whalley and Grant, 1994). Many of these problems were found to be due to the use of insufficient amounts of extractant, i.e. to stoichiometric problems, and 0.1 M hydroxylamine hydrochloride was found to be the most effective and selective extractant for Mn (Neaman et al., 2004). However, it must be used alone, without adding acid to lower the pH, which leads to co-extraction of Fe.

We followed the recommendations outlined in Neaman et al. (2004) for designing the extraction procedure. The initial focus was on using sufficient hydroxylamine. Based on our previous experience (Vázquez et al., 2020; Real et al., 2021), a concentration of 50 mg/g Mn (d.w.) is substantially higher than the typical values observed in moss apical 5 cm segments. In addition, we used a dry/fresh weight ratio of 0.20, which was once again higher than our recorded values (0.12–0.15). Therefore, extraction of up to 50 mg of Mn from 5 g of fresh moss can be expected. We assumed that all of the Mn in the sample would be Mn(IV) because its reduction consumes more hydroxylamine than Mn(III) (Neaman et al., 2004). Under these conditions, a volume of 32 mL of 0.1 M hydroxylamine would be sufficient to reduce all the Mn in the sample. However, as this volume of extractant may be insufficient to fully submerge 5 g of 5 cm moss apices, particularly if the plants are robust, we used 75 mL of extractant.

Extraction for a sufficiently long time is crucial for successful extraction (Neaman et al., 2004). However, longer extraction times may lead to resorption of elements by moss tissue (Vázquez et al., 1999b; Tipping et al., 1985). We determined the optimal extraction time by monitoring the generation of N_2 gas bubbles during hydroxylamine oxidation. In trial tests, we noted that gas production stopped after 10 min, and we therefore extended the extraction time by 5 min. During examination of the extracted material under a dissecting microscope, we also noted that this procedure eliminated the dark precipitates on the moss surface without any apparent effect on the plant (no discoloration or other symptoms of damage).

In summary, 5 g (f. w.) of previously washed moss tissue was placed in plastic containers with 75 mL of 0.1 M hydroxylamine chloride, and extracted for 15 min, with occasional agitation. The extractant was then discarded, and the moss was partly dried by blotting the samples between two sheets of filter paper. The whole segments were cut to produce apical and basal segments, and the samples were dried, ground and the heavy metal contents were determined as outlined above.

2.5. Statistical analysis

2.5.1. Experimental design

The experiment was initially designed as a completely balanced multifactorial ANOVA, to study the effect of the following factors on the element content in the moss tissues: Sampling site (three levels: SS1–SS3); Segment (two levels: 0.0–2.5 cm = AS; 2.5–5.04 cm = BS) and Mn extraction (two levels: extracted and non-extracted material). We excluded data from SS4 because the environmental conditions (alkaline water) were different from those in the other SSs. Some differences between this and the other SSs (e.g. Mn contents two orders of magnitude lower) were so obvious that the inclusion of the data from SS4 would yield statistically significant differences without adding new information and would complicate interpretation of the results by creating interactions between factors.

For each combination of factors, three replicates were used — each from one section of each sampling station. The planned number of data points for each of the twelve elements was therefore $3 \times 2 \times 2 \times 3 = 36$.

We used the R language for the statistical analysis and graphical presentation of the results (R Core Team, 2020).

2.5.2. Data distribution

Heteroscedasticity was observed during the initial exploratory analysis of the data. Moreover, we found significant linear relationships between the mean and the standard deviation of the groups defined by the factors in the analysis, for Cr, Mn, Ni, Zn, Cd and Ba (Table 1). This characteristic is specific to the gamma distribution (Johnson et al., 1994). The properties of this distribution are consistent with the nature of concentration data because the range is $(0, +\infty)$, not $(-\infty, +\infty)$ as in the Gaussian distribution. Moreover, Ott (1995) showed that this is the expected distribution when some solution suffers a (finite) series of random dilutions, as occurs after e.g. release of pollutants into a river.

Data with gamma distributions are intrinsically heteroscedastic because the mean and variance increase together. This violates the assumptions of standard (Gaussian) ANOVA and inflates the Type I error of the tests (Underwood, 1997). A straightforward solution is to use the Generalised Linear Model (glm), which can handle gamma-distributed

Table 1
Parameters, and their significance (*t*-tests), for the regressions of standard deviations on the means of the factor combinations included in the experiment (n = 14).

Element	Intercept		Slope		R^2
	Value	*p*	Value	*p*	
Al	300.252	0.056	0.107	0.112	0.171
Cr	−0.067	0.807	0.357	0.011	0.381
Mn	10.796	0.942	0.198	<0.001	0.624
Fe	797.862	0.002	0.056	0.311	0.073
Ni	−0.585	0.549	0.237	<0.001	0.781
Cu	3.031	0.010	−0.070	0.223	0.104
Zn	−0.522	0.922	0.148	0.003	0.491
As	0.404	0.094	0.122	0.188	0.120
Cd	0.074	0.015	0.042	0.002	0.502
Ba	2.075	0.479	0.094	<0.001	0.687
Hg	0.005	0.135	0.039	0.387	0.054
Pb	−0.378	0.049	0.396	<0.001	0.923

data (McCullagh and Nelder, 1989; Faraway, 2006). We used the R functions *glm*, to perform the calculations, and *anova*, to extract the test results as ANOVA-like tables. *F* significance tests were appropriate in this case, as the response variable was continuous. Note that in glm, deviance is the parameter tested, not variance, and we will refer to this analysis as ANODEV hereafter.

The right-skewness of the gamma distribution led to unexpected results during the study of element extractability. We calculated the ratios between element concentrations after and before Mn extraction and used the mean values for each factor combination (n = 3 data) to reduce data noise. Surprisingly, extractability >1 was common for some elements. We suspected the distribution of the ratios as a possible cause of this result, and we carried out simulations to test this idea (we did not find any reference to the closed form of such distribution in the relevant literature). We generated n = 10,000 pairs of groups of three random data from gamma distributions with mean $\overline{m} = 10$ and varying from 1 to 30 in 0.5 steps (using the R function *rgamma*). These two parameters define the shape of the distribution, but as k becomes smaller, the distribution becomes more skewed to the right. The results are shown in Fig. 2. The distributions were also right-skewed, with means always greater than 1, but approaching this value as k increased. When k = 6.5 (which is close to our observed values), the simulation yielded $\overline{m}$ = 1.06, but the probability envelopes show that larger values are likely. Moreover, the simulation revealed that the median remained very close to 1 and was independent of k.

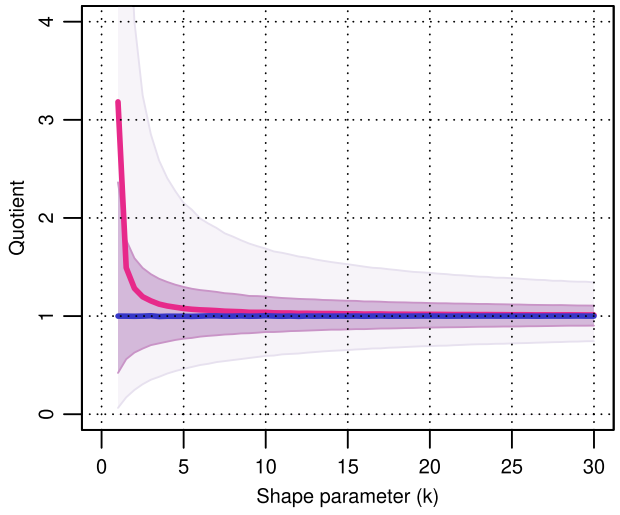

Fig. 2. Results of the simulation of the distribution of the mean ratio extracted/non-extracted (n = 3) for data with a gamma distribution. The mean (red line), median (blue line) and the envelopes including 25–75 %, and 2.5–97.5 % of the data are shown. The means of the simulated distributions were the same, $\overline{m}$ = 10, and the shape parameters ranged from k = 1 to k = 30, in 0.5 unit steps. For each k, n = 10,000 quotients were simulated.

3. Results

3.1. Element concentrations

The concentrations determined in the non-extracted samples are shown in Table 2. The concentrations were low but differed between the SSs. Considering the SSs with near-neutral water, the concentrations of Al, Fe, Cr, Pb and particularly Ni were highest in SS3, while the concentrations of Cu and Zn were highest in SS1. For As, Ba, Cd and Hg, the SS with the highest concentrations depended whether AS or BS was considered. In all SSs, the concentrations of most elements were higher in the basal segments (i.e. in the older tissues) than in the apical segments. The concentrations of Cu and Hg were slightly lower in the basal segments or were the same in both segments.

In SS4, the concentrations of Mn were at least one order of magnitude lower than in the other SSs, and the concentrations of Fe and Ba were also low. The concentrations of Cd and Pb were higher than in other SSs. In addition, in SS4 the element concentrations were higher in the apical segments than in the basal segments.

3.2. Results of the ANODEV

The results of the ANODEV are summarized in Table 3 (only SS1–SS3 were included, see Material and methods section). The *p*-values (*F* tests) indicate the significance of the effects of the factors and of their interactions on the concentrations of the elements. Fig. 3 shows the interaction plots for the three factors in the analysis.

Regarding the effects of the factors the following results were obtained: a) SS was significant for all the elements. However, the SS with the highest concentrations depended on the element considered. Given the different environmental settings and potential pollutant sources in the various SSs, the heterogeneity of results was not surprising. b) The concentrations of all elements except for Cr, Cu and Hg were significantly higher in the basal segments than in the apical segments. c) Mn extraction yielded significantly and consistently lower concentrations of Mn, Zn, Cd, Ba and Ni (less clear) in all SSs. Higher concentrations of Fe, Cu, Hg and Pb were observed after extraction in SS1 only, but this was sufficient to qualify the effect of extraction as significant for Cu and Pb. The concentrations of Al, Cr and As (this showed minimal changes) varied inconsistently, and the effect was not significant.

Interpretation of the factor effects was difficult due to interactions that varied with the element (Fig. 3). The SS:Segment interaction was caused by the different order of the SSs pairs of means for AS and BS (ex.: As-AS: SS2 > SS3 > SS1; As-BS: SS3 > SS2 > SS1). The interaction was significant for Mn, As, Cd, Ba and Pb.

The SS:Extraction interaction was significant for Al, Cr, Ni, Zn and Ba. Again, the ranking of the SSs changed after extraction. The concentrations of Al, Cr and (to a lesser extent) Ni in some samples were lower after the extraction but higher in others. The concentrations of Zn and Ba were consistently lower after extraction, but the ranking of the SSs nevertheless differed.

The Segment x Extraction interaction was only significant for Mn and Zn. These elements were the most strongly extracted, and the concentrations converged to a narrow range after extraction. The interaction was due to this factor and the initial higher concentrations in BS.

3.3. Performance of the extraction procedure

Comparing the effect of extraction for the different elements requires the use of units other than absolute concentration values. For this purpose, we determined the extraction ratios by calculating the quotient of the concentrations with and without extraction. The data are

Table 2
Mean element concentrations in non-extracted samples ($\mu g\ g^{-1}$, n = 3) in the apical (AS) and basal (BS) shoot segments from each sampling site.

Elem.	SS1		SS2		SS3		SS4	
	AS	BS	AS	BS	AS	BS	AS	BS
Al	1004	1902	1409	1748	3831	3775	1343	996
Cr	0.65	0.91	1.50	1.78	3.58	4.13	2.90	1.41
Mn	2739	8798	4258	6689	1739	6683	143	121
Fe	2329	4345	2845	3751	4472	6918	1937	1404
Ni	4.4	11.8	9.1	15.2	27.8	85.3	3.9	2.8
Cu	14.4	12.0	8.6	8.0	17.4	17.1	29.5	22.5
Zn	173	277	61	81	117	176	172	150
As	0.77	2.01	2.47	3.66	2.08	4.27	2.76	1.79
Cd	0.28	0.71	0.47	0.60	0.58	1.16	7.53	3.01
Ba	193	404	225	290	57	87	13	11
Hg	0.08	0.06	0.04	0.04	0.04	0.04	0.11	0.09
Pb	0.82	2.05	0.85	1.08	1.32	1.66	12.46	10.80

Table 3
Summary of the multifactor GLM-ANOVA analysis. The *p*-values for the *F* tests are shown and significant values ($p \leq 0.050$) are highlighted. The factors were Sampling station (SS; three levels, SS4 was not included), Segment (Sm; two levels) and Mn extraction (Ex; two levels). The total number of data points in each analysis was N = 36, and the number of replicates for each factor combination was n = 3.

Elem.	Factors			Interactions			
	SS	Sm	Ex	SS:Sm	SS:Ex	Sm:Ex	SS:Sm:Ex
Al	**<0.001**	**0.011**	0.233	**0.004**	**0.012**	0.972	0.391
Cr	**<0.001**	0.465	0.695	0.600	**<0.001**	0.400	0.241
Mn	**0.005**	**<0.001**	**<0.001**	**<0.001**	0.337	**0.001**	0.460
Fe	**0.001**	**<0.001**	0.276	0.350	0.420	0.721	0.877
Ni	**<0.001**	**<0.001**	**0.011**	**<0.001**	**0.017**	0.936	0.895
Cu	**<0.001**	0.168	**0.011**	0.550	0.143	0.927	0.775
Zn	**<0.001**	**<0.001**	**<0.001**	0.507	**0.022**	**0.034**	0.570
As	**<0.001**	**<0.001**	0.836	**0.003**	0.862	0.996	0.922
Cd	**<0.001**	**<0.001**	**<0.001**	**0.018**	0.584	0.718	0.609
Ba	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.842	**0.033**
Hg	**<0.001**	0.226	0.210	0.051	0.859	0.782	0.977
Pb	**<0.001**	**<0.001**	**0.002**	**0.003**	0.071	0.112	0.203

summarized as the means of the n = 3 samples for each factor combination and are included in Table 4.

The results showed that the hydroxylamine solution extracted most of the Mn. The proportions of the remaining Mn were below 0.30, except for SS4. Inspection of the data revealed that the proportion is dependent on the initial Mn contents. Therefore, the basal segments of SS1–SS3 had the lowest remaining proportions, followed by the apical segments and the SS4 samples. For SS1–SS3 the range of concentrations (individual data, not means) changed from 1407 to 9424 to 326–1662 $\mu g\ g^{-1}$, and for SS4 from 114 to 154 to 48–67 $\mu g\ g^{-1}$.

3.4. Coextraction of other elements

Another important characteristic of the extraction method is its specificity, i.e. its capacity to extract Mn leaving Fe unaffected. We used only hydroxylamine without further acidification to enhance the specificity. The ratios in Table 4 confirm that the extraction was selective for Mn, as the concentrations of Fe and Al (another hydroxide-forming element) remained close to those in the non-extracted samples. This is consistent with the results of the ANODEV, where Extraction was not a significant factor for these two elements. In SS4, however, ≈50% of the Fe and Al were coextracted with Mn in SS4.

The effect of Extraction was also significant for Ba, Cd, Ni and Zn (Table 4). This effect was not observed in SS4.

In SS1, the concentrations of Fe, Cu and Pb were higher after extraction in both apical and basal segments (corresponding with the results of the ANODEV). The extraction also yielded higher concentrations in the other SSs, but the effect was much more moderate, hence the significant SS x Extraction interaction. Note that the effect of Extraction was significant only for Pb and Cu, but not for Fe. A similar pattern was observed for Hg, but the increases in SS1 were smaller and this resulted in non-significant effects for Extraction and the SS:Extraction interaction.

Finally, Cr and As, which were not affected by the extraction process in SS1–SS3, were coextracted in large proportion in SS4.

4. Discussion

4.1. Element concentrations

The concentrations of all elements were low. For Cr, Cu, Ni, Pb and Zn the mean concentrations in the non-extracted samples were lower than the background values proposed by Carballeira et al. (2002) for the same geographic area (the other elements were not analysed). As a result, the differences in concentrations in SS1–SS3 were generally small. The only exception was Ni, which was present in SS3 at concentrations 5 times higher than in the other SSs, while remaining below the background value. These differences were expected, as the SSs were selected to represent the variation in conditions in the rivers in the study area.

The concentrations of Cd and Pb were higher in SS4 than in the other SS. This can probably be attributed to the composition of the calcareous rocks, as the concentrations of these elements in soils developed on this type of rock exceed regional reference levels (Macías-Vázquez and Calvo de Anta, 2008). However, the greatest difference was observed for Mn, the concentrations of which were one order of magnitude lower in SS4 than in the other SSs owing to the higher pH of the water in SS4.

The comparison between apical and basal segments showed that most metals were present at higher concentrations in the basal parts of the shoot in SS1–SS3. This trend has been observed in other studies and has been attributed to the deposition of greater amounts of Mn and Fe

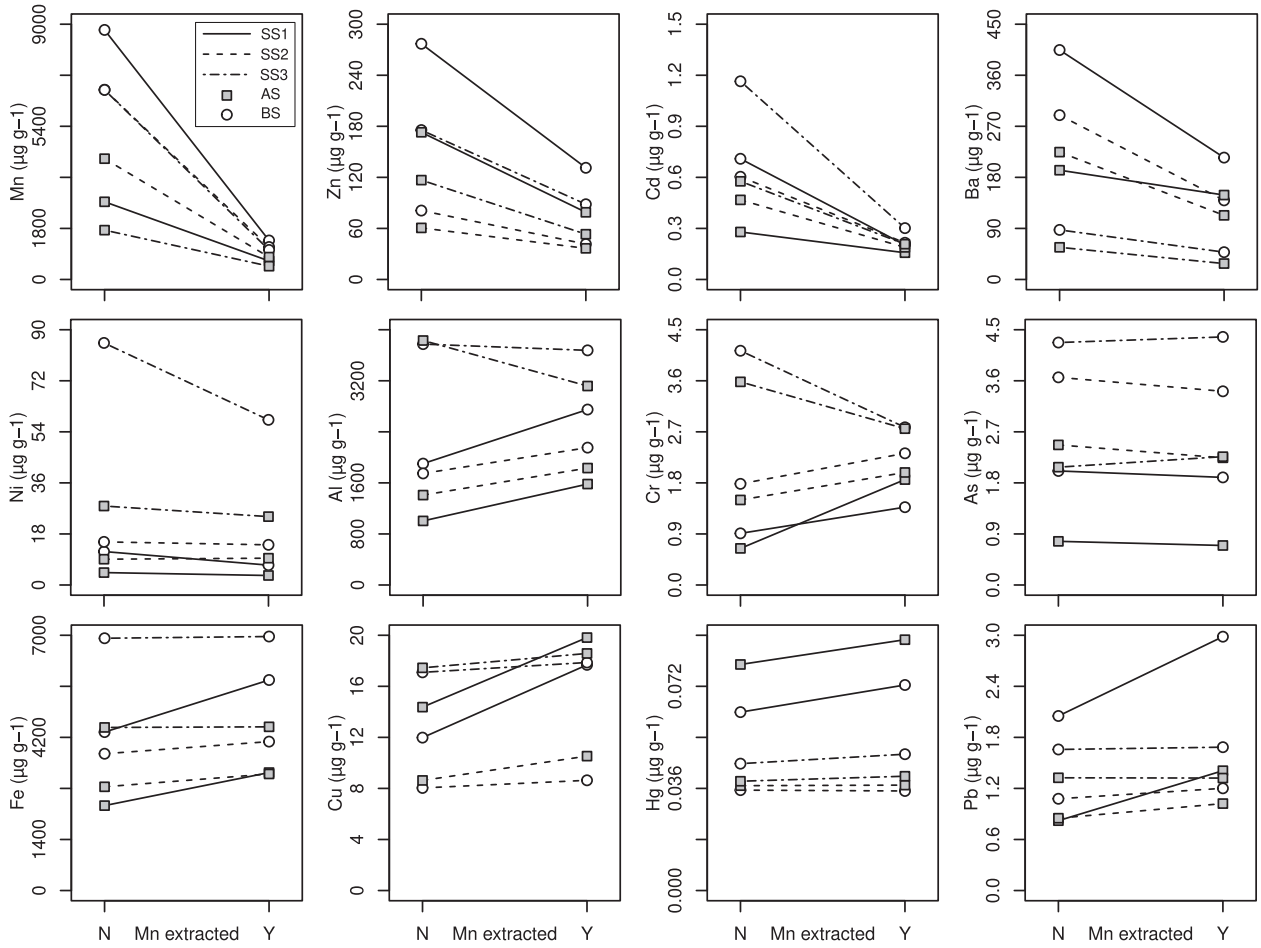

Fig. 3. Interaction plots for the ANODEV analysis. The values are the means (n = 3) of each factor combination. The different SSs are indicated by different types of lines, and the type of segment by different symbols (see insert in first graph: AS = apical segment; BS = basal segment). The position on the horizontal axis separates extracted (Y) and non-extracted (N) samples.

Table 4
Proportion remaining after extraction (quotient between the extracted/un-extracted concentrations). The values are the means of 3 replicates. AS: apical 2.5 cm; BS: the following 2.5 cm).

Elem.	AS				BS			
	SS1	SS2	SS3	SS4	SS1	SS2	SS3	SS4
Al	1.57	1.30	0.81	0.54	1.45	1.23	0.97	0.54
Cr	2.88	1.32	0.77	0.40	1.51	1.30	0.67	0.61
Mn	0.24	0.19	0.27	0.44	0.16	0.17	0.16	0.43
Fe	1.39	1.12	1.00	0.53	1.33	1.09	1.01	0.56
Ni	0.77	1.04	0.87	0.76	0.59	0.93	0.68	0.83
Cu	1.38	1.22	1.06	1.07	1.48	1.07	1.05	1.02
Zn	0.46	0.60	0.46	0.64	0.47	0.51	0.50	0.63
As	0.91	0.91	1.09	0.42	0.94	0.93	1.02	0.50
Cd	0.56	0.40	0.36	0.56	0.29	0.36	0.26	0.52
Ba	0.77	0.50	0.49	0.61	0.53	0.48	0.55	0.62
Hg	1.11	1.01	1.05	1.03	1.15	0.99	1.07	1.06
Pb	1.72	1.20	1.00	0.95	1.45	1.11	1.02	0.86

oxides in the most basal parts of mosses (Whitton et al., 1982; Martínez-Abaigar et al., 2002; Vázquez et al., 2020). However, the opposite pattern was observed in SS4, where the highest concentrations of most elements, including Mn, were detected in the apical segments.

4.2. Performance of the extraction procedure

The performance of the Mn extraction procedure will be discussed considering three potential and interrelated problems: extraction power, specificity and resorption of the extracted Mn.

Regarding the extraction power, only 16–27 % of the initial Mn content in samples from SS1–SS3 remained (see Table 4). Mn was extracted more quickly from moss samples (15 min) than from soils (Neaman et al. (2004) observed that up to 2 h were necessary to obtain 85 % of the Mn from some soils).

The amount of hydroxylamine used can dissolve up to 100 mg g^{-1} Mn (d.w.). However, this concentration was not reached in any of the samples, with the highest concentration measured being 9.4 mg g^{-1} Mn. There are several explanations for the residual fraction remaining in all samples. First, part of the Mn was inside the cells, as it is a micronutrient with important biochemical roles (e.g. in photosynthesis). However, the concentrations remaining after extraction varied between 48 (in one of the SS4 samples) and 1662 ppm (36-fold increase). The differences in residual Mn cannot only be attributed to physiological causes.

Part of the Mn could be co-precipitated with the Fe oxides and thus be inaccessible to the extractant. However, the Fe and Mn concentrations after extraction were poorly correlated ($r^2 = 0.228$, $p = 0.045$). Therefore, although the Fe-bound fraction exists, it seems to account for a variable fraction of the residual Mn. More detailed research is needed to determine the relationship between Fe oxide deposits and residual Mn. In this regard, a two-step extraction procedure may be useful: extraction with hydroxylamine (Mn oxides) followed by acidified hydroxylamine (pH < 3 — Fe and Mn oxohydroxides). We will discuss the use of double extraction later.

The residual Mn could be a fraction of Mn that was reabsorbed by the cell wall after dissolution. However, García-Seoane et al. (2023) found that the net charge of the cell wall of *F. antipyretica* is positive at pH < 4.51. Therefore, as the extraction medium is acidic (pH 3.6, see Neaman et al. (2004)) the moss surface would not bind cations. The reduction of Mn implies the conversion of hydroxylamine into N_2 and water with consumption of H^+ and an increase in pH. However, 98.5 % of the hydroxylamine would have to be consumed in order to increase the pH above 4.51. Although we did not measure the final pH of the solution, it can be assumed to have remained well below 4.51 given the excess hydroxylamine used.

Lastly, it is conceivable that some of the Mn oxides aged, resulting in increased crystallinity and rendering them more difficult to extract. This process has been observed in soils, for example Chao (1972) found that the Mn extraction efficiency of hidroxylamine ($+HNO_3$, pH 2) was 50 % in highly weathered soils, while it reached 85 % in younger soils. The observed trend of an increase in the residual fraction from AS to BS segments is in line with this hypothesis.

Given that these processes are not mutually exclusive, they probably occur together; however, the data obtained in the present study do not allow us to evaluate the relative importance of each.

Regarding the specificity, the extractant did not extract substantial amounts of Fe from SS1–SS3. In fact, the extractability of most Fe residual fractions was >1 (Table 4). This apparently impossible outcome can be explained by the gamma distribution of the data and the determination of element concentrations in different samples (extracted and non-extracted). The same pattern was observed with Cr and Pb.

In SS4, 50 % of both Mn and Fe were co-extracted. Given the alkaline pH of the water, and considering the equilibria in Fig. 1, we believe that both elements may be deposited as carbonates. If so, the extractant would be acidic enough to dissolve them and also extract Fe, Mn and other elements.

Finally, the extraction did not appreciably alter the appearance of the moss, apart from the loss of the dark deposits on its surface. No symptoms of chlorophyll degradation, turgidity loss or other damage were observed, despite the acidity of the extractant solution. It is possible that the short extraction time prevented tissue degradation.

4.3. Co-extraction of other elements

After establishing that a high proportion of Mn had been deposited on the moss surface, the next question was to determine which other elements were associated with it. According to the ANODEV findings (refer to Table 3), Extraction was a significant factor for seven elements, while only Ba, Cd, Ni, and Zn were definitely co-extracted with Mn. The overall mean fractions extracted were Cd = 0.67, Zn = 0.50 and Ba = 0.54 (calculated from Table 4, including AS and BS data but excluding SS4). The pattern was less clear for Ni, as SS2 concentrations were not affected by the extraction, and the overall proportion extracted was only 0.19. Previous studies of the absorption capacity of Mn oxides showed that they have a strong affinity for Cd (Dong et al., 2003b; Zhou et al., 2015; Feng et al., 2007; Meng et al., 2009), Ni (Gray et al., 2001; Gray and Hill, 1995; Haak and Warren, 2007) and Zn (Mori et al., 2018; Zhou et al., 2015; Feng et al., 2007). We did not find any information on Ba absorption.

The ANODEV analysis also revealed a significant effect of Extraction on Cu and Pb concentrations. However, the interaction plots (Fig. 3) showed that the contents were only different in SS1. These elements could be associated with Fe, which followed a similar pattern. These results differ from those reported in previous studies, which found that Mn oxides have a high capacity to absorb Pb and Cu (McKenzie, 1980; Zhou et al., 2015; Feng et al., 2007). However, some studies did not separate the effects of Fe and Mn oxides (Dong et al., 2003a).

In SS4, the extraction ratio was similar for most elements (40–60 %), except for Cu, Hg, and Pb, which were not extracted at all. There was minimal change in the ratio between shoot segments (see Table 4). The extractability of Fe and Mn in SS4 were similar, in contrast to the other SSs. This behavior could be explained by the important role of carbonates in the precipitation of other elements, as commented above.

4.4. Use of the technique

The previous discussion showed that the proposed method is suitable for the selective extraction of Mn oxides and associated elements. The next question is how the method could be incorporated in biomonitoring studies to improve the estimation of pollutant concentrations, or to reveal bias in interpreting total concentration data. Note that this study was designed to investigate the performance of the technique, not to address such questions. Nevertheless, even with the limited data produced, some applications and related results can be suggested at this point.

The obvious application of the method is to correct the bias in data caused by precipitation of Mn. Any increase in Mn deposition, caused by variations in the Eh and/or pH of the water, might cause a parallel increase in the concentrations of the co-precipitating elements. However, the opposite is also possible: decreases in the Mn deposition rate may (over-)compensate for increases in element concentrations. In the first case, the results could be misinterpreted as increases in pollution level and in the second, real increases could be overlooked.

To prevent such misinterpretations when comparing samples, the effect of Mn must be corrected. This can be done by converting all the Mn concentrations to a common value and modifying the concentrations of the other elements correspondingly. Calculating the element/Mn ratio in the extracted fraction enables estimation of how much of an element the extra Mn would add (or subtract, depending on the reference Mn value selected).

We applied the correction to the Cd data from SS1-SS3, including both apical and basal segment data. Fig. 4 shows the original and corrected concentrations, supposing that all samples contained 10,000 $\mu g\ g^{-1}$ Mn (the maximum value in the original data was 9424 $\mu g\ g^{-1}$ Mn). We assumed that all the supplementary Mn was extractable.

The figure shows several remarkable effects of the correction. First, in SS1 and SS2, the variability in the data decreased, along with the differences between AS and BS. The overall difference between the two SSs also almost disappeared. However, Cd concentrations in SS3, which were already higher than those in the other SSs for uncorrected data,

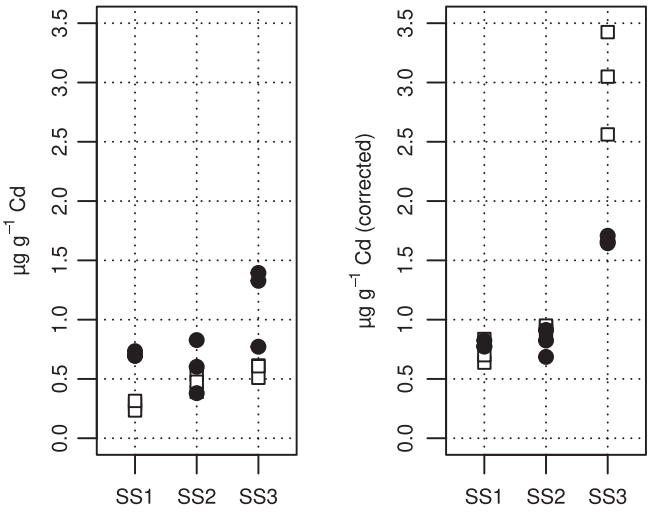

Fig. 4. Left: concentrations of Cd in non-extracted samples from sites SS1 to SS3. Right: concentrations corrected with the ratio Cd/Mn in the extracted fraction for each sample and calculated for a total extractable Mn content = 10,000 $\mu g\ g^{-1}$. Squares correspond to apical segment (AS) data and circles to basal segments (BS).

increased after correction. This shows that the Mn oxides may be a source of noise in the data and that the correction may improve the quality of the data in most studies.

The most interesting effect of the correction involved the differences between AS and BS data. The fact that the apical part of the stem is formed by young tissue explains the lower (uncorrected) contents of most of the elements, and we therefore expected that the correction would compensate for the age-related effect. Indeed, this was the case for SS1 and SS2, but in SS3, the element concentrations were higher in apical segments than in basal segments. This suggests that the concentrations of Cd in water had increased recently in SS3, while they were stable in SS1 and SS2. This was an unexpected finding, which would have been completely overlooked if only uncorrected data were considered. Polluted run-off from the coal mining area upstream of SS3 is a possible source of Cd, but a specific study should be done to confirm this possibility.

4.5. More complex scenarios

In studies involving waters of markedly different characteristics, a high degree of bias could be introduced by Mn oxides. There are at least two different scenarios where this could occur. If the study area includes acidic and alkaline waters, accumulation of Mn in the latter case might be an order of magnitude lower, as occurred in SS4. Such a difference in Mn content would introduce a very high degree of bias in comparisons between samples from acidic and alkaline waters. Moreover, some of the Mn and other elements were deposited as carbonates rather than as oxides in alkaline environments like SS4. The extraction was designed for Mn oxides, not for carbonates, which become solubilized because of the acidity of the hydroxylamine solution and the element/Mn ratios in both environments are not comparable. Therefore, the correction is not meaningful in this situation. Perhaps a more sensible approach would be to use the remaining element content to compare acidic and alkaline samples; however, further research would be required to develop such a procedure.

A possible different scenario would be to study a gradient ranging from very acidic to mildly acidic waters. Such gradients occur, for example, when mine drainage water mixes and becomes diluted by less acidic water. In acidic waters, Mn remains in the soluble state, while Fe oxides precipitate (see Fig. 1). Fe oxides can capture other elements by co-precipitation, like Mn oxides do. Therefore, a shift in the relative abundance of Fe and Mn oxides along a pH gradient is expected. As the elements associated with Fe and Mn oxides may not be the same, relative increases in some elements might be caused by this shift in oxyhydroxide dominance, not by changes in water concentration. There is again a bias problem, but with two sources of bias acting simultaneously.

One possible solution would be to extract the oxides in a pseudo-sequential way. Thus, a sample aliquot could be treated with hydroxylamine to extract the Mn oxides and another could be treated with acidified hydroxylamine to extract both Fe and Mn oxides. The data thus obtained could be used to determine the quantities of each oxyhydroxide and the contents of co-precipitated elements, which could then be used to correct the concentration data.

5. Final remarks

We are confident that this technique can be used to improve the results and interpretation of trace element biomonitoring studies in aquatic environments. The full potential of the technique will only be revealed in future studies, but the data obtained in the present study demonstrate the importance of Mn and its oxides in near-neutral waters. Even if this technique is not applied in future studies, Mn should at least be included among the elements measured to provide some indication of any bias in the data.

Finally, other species of aquatic macrophytes that are used as biomonitors probably accumulate elements in the same manner, and studies with such species would also benefit from the application of this technique.

CRediT authorship contribution statement

Carlos Real: Writing – original draft, Project administration, Investigation, Formal analysis, Conceptualization. **Rubén Villares:** Writing – review & editing, Investigation, Conceptualization. **María Dolores Vázquez:** Writing – review & editing, Investigation, Conceptualization.

Declaration of competing interest

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

Data availability

The authors are unable or have chosen not to specify which data has been used.

Acknowledgements

This work was supported by Ministerio de Ciencia, Innovación 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. Authors would like to thank RIAIDT-USC for the use of its analytical facilities.

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