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Evaluation of Heavy Metal Removal Using *Phragmites Australis* (Cav.) and *Schoenoplectus Californicus* (C.A. Mey.): A Comparison of the Dry Ashing and Wet Digestion Method

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This study presents the evaluation of heavy metal removal using *Phragmites australis (Cav.)* and *Schoenoplectus californicus (C.A. Mey.)* in a laboratory wetland test (10-days). Two digestion methods: Dry Ashing and Wet Digestion to determine the final concentration of heavy metal in the roots and stems of both plants were used. The final concentration of Cu (5.14 μg g^{-1}), Zn (27.34 μg g^{-1}), and Fe (107.91 μg g^{-1}) were determined in the roots of the *Schoenoplectus californicus (C.A. Mey.)*. While in its stems the highest concentration of Pb (1.69 μg g^{-1}) was founded. In *Phragmites australis (Cav.)* the high concentrations of Cu (2.44 μg g^{-1}), Zn (5.22 μg g^{-1}), and Fe (28.10 μg g^{-1}) are found in the roots and Pb (0.70 μg g^{-1}) in the stems. Regardless of the plants studied, the Wet Digestion method was the most suitable pretreatment method for determining Cu and Fe concentrations, while the Dry Ashing method was the best for Zn and Pb.

Keywords: Dry ashing, *Phragmites australis,* Phytoremediation, *Schoenoplectus californicus*, Wet digestion

INTRODUCTION

One of the greatest environmental issues related to the development of mining activity is the generation of Acid Mine Drainage (AMD). The AMD lowers the pH of natural water resources such as rivers and lakes, allowing heavy metals to be easily dissolved. Acid Mine Drainage can also affect soils [1]. As we know, the soil is a basic substrate in terrestrial ecosystems and is the primary basis for agricultural production [2].

Many methods for the treatment of AMD and restoring contaminated soils [3-5] have been developed, being wetlands a good option for heavy metal removal [6-8]. In Fact, phytoremediation through wetlands is a useful passive technique for cleaning up wastes, including metals, pesticides, crude oil, polyaromatic hydrocarbons, and landfill leachate [9].

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Phytoremediation is applied to sites with surface contamination from organic compounds and metal contaminants [10]. Which can intervene in any of the following actions: phytotransformation, bioremediation of the rhizosphere, phytostabilization, phytoextraction, or rhizofiltration [11,12]. For metal contaminants, plants show the potential for uptake and recovery of contaminants in above-ground biomass (phytoextraction), filtering metals from water into root systems (rhizofiltration), or stabilizing waste sites through erosion control and evapotranspiration of large amounts of water (phytostabilization) [9].

Green plants growing in wetlands and thus their associated microbiota seems to be a good technique to remove toxic pollutants in industrial effluents [13]. However, the efficiency of the plants in the remediation of these contaminants depends on several factors such as hydraulic retention time, type of plant, and concentration of contaminants, among others. On the other hand, the ideal plants must be resistant to climatic changes; be able to

tolerate low levels of nutrients, and absorb more pollutants than plants under normal conditions [7]. Depending on the specific characteristics of the medium, different authors analyzed the behavior of various local species to remove heavy metals. Among the reported results we have the use of *Cyperus esculentus* in the absorption of zinc and cadmium in freshwater sediments [14], *Eichhornia crassipes* in coal mine effluents [15], *Pista stratitoes* for removal of various heavy metals such as Cu, Cr, Fe, Mn, Pb and Zn [9], *Typhia* to remove copper [16,17] and *Vetiveria zizanioides* to reduce the concentration of heavy metals such as Cu, Fe, Mn and Zn [18].

The versatility of passive wetland-based methods has produced a large number of investigations regarding the use of different bioaccumulative plants [15,17,19-21]. Thus, the use of *Phragmites australis (Cav.)* and *Schoenoplectus californicus (C.A. Mey.)* represents an interesting alternative for the remediation of AMD [22-28].

Metals can be transferred from soil or water into plants through the roots in the form of dissolved ions through a series of complex processes [29]. In fact, heavy metals can be accumulated in the root, stem, leave, or fruit of diverse plants [20,27,30-33]. It becomes necessary to obtain accurate and reliable data on the concentrations of heavy metals in each part of the plant [34].

The determination of heavy metals in organic-plant material is carried out by flame atomic absorption spectroscopy (FAAS) [35]. Furthermore, the advantage is that FAAS is a simple method and has a high sensitivity [35]. However, prior to analysis by FAAS, it is necessary to digest the samples adequately [36-39]. Dry Ashing (DA) and Wet Digestion (WD) methods have been developed to realize the chemical analysis of heavy metals in human hair and nails [40], animals [41,42], organic residues [43], microorganisms [44], fruits [45], food [46] and plants [47-49].

The chemical analysis of metal in the vegetable samples involved two main processes: acid or wet digestion of samples. In both methods, the organic chemical integrity of the plant tissues is disintegrated into inorganic and molecular forms, which is essential for the estimation of metal elements in plant samples; and the estimation of metals in the acid-digested samples [43,46,49]. Therefore, there is a great need to study the effect of different digestion methods on the extraction of heavy metals from plants [50].

Here, we report a comparison of heavy metal concentrations content in the root and stem of *Phragmites australis* and *Schoenoplectus californicus* determined by FAAS after following DA and WD methods.

MATERIALS AND METHODS

Plant Samples

The samples of *Phragmites australis (Cav.)* and *Schoenoplectus californicus (C.A. Mey.)* were obtained from the south of Lima, Peru. The plants were carefully treated and placed into laboratory wetlands cells [51,52]. The cells contain gravels (30 mm to 40 mm diameter) as substrate [27].

Chemicals

Copper sulfate pentahydrate ($CuSO_4.5H_2O$), zinc sulfate pentahydrate ($ZnSO_4.5H_2O$), lead sulfate pentahydrate ($PbSO_4.5H_2O$), iron sulfate pentahydrate ($FeSO_4.5H_2O$), sodium nitrate ($NaNO_3$), ammonia (NH_3) and nitric acid (HNO_3) were purchased from Sigma-Aldrich Peru (Lima, Peru). All chemicals were of analytical purity and they were used as received without further purification. All aqueous solutions and dilutions were prepared with Milli-Q water (18 MΩ cm). The purification system (Millipore, Darmstadt, Germany) is located in our laboratory. Finally, 1000 mg l^{-1} standard solutions of Copper, Zinc, Lead, and Iron for the procedure in the Atomic Absorption Spectroscopy (AAS) were purchased from Merck Peru (Lima, Peru).

Effluent Preparation

A quaternary solution containing the most common metals in the AMD (Cu-Zn-Pb-Fe) was prepared [53-57]. A certain amount of pentahydrated metal sulfates was dissolved in 1L of distilled water to obtain a stock solution of metal ions (1000 mg l^{-1}). The necessary dilutions were then made to obtain the desired initial concentration of metals (see Table 1). The preparation was executed according to the methods indicated by *Al-Subu et al.* [58] and *Paksamut et al.* [59].

Equipment

A grinder (Bosh, MKM6003) and an oven (Heraeus, UT6) to mill and reduce the humidity in samples were used respectively. To weigh the samples, a 4-decimal precision

Table 1. Instrumental Conditions of the Metal Analysis by FAAS

Parameters	Zn	Fe	Pb	Cu
Wavelength (nm)	213.9	248.3	283.3	324.8
Slit (nm)	1	0.2	1	0.5
Lamp current (mA)	9	15	10	8
Calibration range (mg l^{-1})	0.2-1	1-5	2-10	1-5
Flame composition	Air/acetylene			
Oxidant presure (bar)	0.758			
Atomizer	Standard Burner			
Measure mode	Absorbance			
Concentration of quaternary solution (mg l^{-1})	0.018	0.013	0.015	0.016

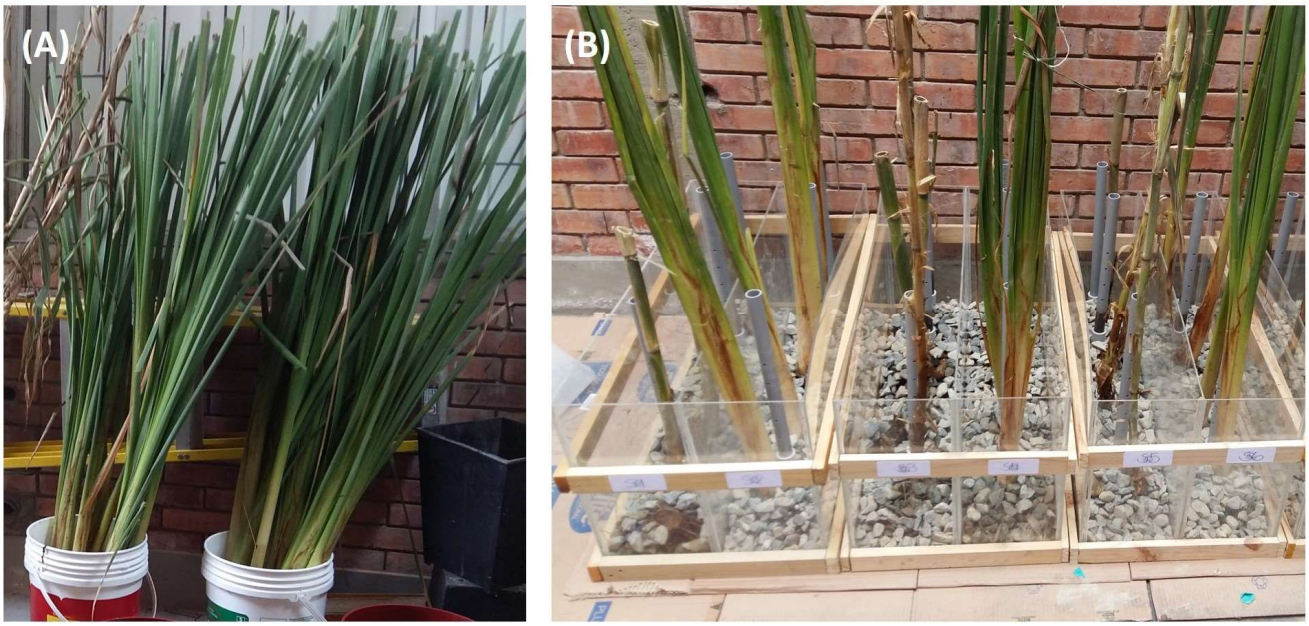

Fig. 1. a) Plant conditioning, b) Plant arrangement in each laboratory cell as constructed wetland.

analytical scale (Sartorius, CP225D) was used. Two thermostat hotplates (Gallenkamp, Hpl600 050E) were used for wet digestion. A compact muffle furnace (Carbolite, LMF3) was used for converting the samples into ashes. The final concentration of heavy metals in each part of the plant was analyzed by atomic absorption spectrometry (Atomic Absorption Spectrometer, Varian AA 220) using an air/acetylene flame [60].

Samples Preparation

Both plants, *P. australis* and *S. californicus* were carefully placed into the cells (Fig. 1). Then, a solution containing heavy metals (Cu-Zn-Pb-Fe) was added [56]. At the end of the bioremediation experiment (10-days), the plants were removed from the wetland. Then they were washed and arranged on paper towels to ensure uniform exposure to the air. After a week, 14 sample plants were

selected. Then, the roots, stems, and fruits of each plant were carefully separated. The stem and roots of each plant were settled in beakers and placed in an oven at 100 °C to eliminate the humidity. After 2 days, the plants were placed in a desiccator. Then, by using a grinder the samples were pulverized [27].

Dry ashing method. The samples were prepared by adapting the No. 17365-94 dry ashing method of the Bulgarian State Standard [61] and the No. 3.007a standard method of the AOAC [62]. Then, 1g of dried plant sample was weighed into a porcelain crucible and then calcinated at 500 °C for 12 h in a muffle [63-65]. In order to dissolve the inorganic material, the ash obtained was placed in a glass beaker with 15 ml of HNO_3 (1.0 M). The beaker was heated for 30 min in a hot plate where the temperature was increased to 100 °C. The residue was filtered into a 25 ml volumetric flask using filter paper (Whatman, No. 42); the volume was completed with HNO_3 (1.0 M) [66].

Wet digestion method. For the wet digestion (WD) method, the EPA 3050B method was modified [67]. In fact, 1g of dry plant sample was placed in an Erlenmeyer flask. Then 10 ml of HNO_3 (1.0 M) was added. A digestion procedure was done for 12 h approximately [36,68,69]. Once the time was finished, the flask was heated for 2 h at 60 °C. Then the temperature was increased to 100 °C during 2 h. The remaining solid material was eliminated by filtration with filter paper (Whatman, No. 42). The solution was collected in a volumetric flask (25 ml). The volume was completed with Milli-Q water.

Heavy Metal Concentration

The concentration of metals was determined by the FAAS. To establish the accuracy of the measurement of metal concentration with this method, blanks and standards were prepared based on the standard procedures [36,70,71]. The detection limits were 0.06 mg l^{-1} for Fe, 0.03 mg l^{-1} for Cu, 0.01 mg l^{-1} for Zn and 0.1 mg l^{-1} for Pb. During the analysis, a blank solution with 2 ml of the 0.50% aqueous HNO_3 was prepared. Furthermore, with the dilution of 1000 ppm of the stock with 0.50% aqueous HNO_3, three standard solutions (100 ml) for each metal were prepared. The blank, standard (from lowest to highest concentration), and sample solutions were placed in that order on the autosampler. In the end, all absorbance results were registered. Table 1 shows the instrumental conditions.

Statistical Analysis

In order to compare the final concentrations of Cu, Zn, Pb, and Fe in the roots and stems of each species using the DA and WD method, a two-way Analysis of Variance (ANOVA) was used. The two-way ANOVA examines the influence of two different categorical independent variables on one continuous dependent variable [72-74]. The analysis involves the calculation of the F statistic and it is compared with the critical value of a Snedecor F distribution with the confidence level assigned for the test [75]. The F statistic is a test used to assess the explanatory power of a group of independent variables on the variation of the dependent variable. The F statistic is a ratio of two variances (the variance of the means of the groups and the average of the variance within the groups). In general, an F statistic is a ratio of two quantities that are expected to be approximately equal under the null hypothesis. The analysis considered a confidence level of 0.95 ($P < 0.05$), using Minitab software. The Student's t-test (t-test) method was used to determine if there is a significant difference in heavy metal concentration when the wet digestion method and dry digestion method were used. Student's t-test was employed to estimate the significance of values at a probability level of 95% [76].

$$\bar{X}_1 - \bar{X}_2 = ts\sqrt{\frac{N_1+N_2}{N_1N_2}} \quad (1)$$

Where $\bar{x}_1$ and $\bar{x}_2$ are the samples mean, n_1 and n_2 are the sample size, and s is the standard deviation. On the other hand, the comparison of the standard deviations between both methods can be a problem due to the different values in the removal [47]. Therefore, it is necessary to evaluate the relative standard deviation (RSD), according to the following formula:

$$RSD = \frac{Standard\ deviation}{Average} \quad (2)$$

The t-test and ANOVA are statistical methods used in the testing of hypotheses for the comparison of means between the groups [77].

RESULTS AND DISCUSSION

Table 2 and Table 3 present the heavy metal

Table 2. Heavy Metal Concentrations (μg g^{-1}) in *P. australis* and *S. californicus* Determined by FAAS after Digestion Using Dry Ashing Method

	P. australis				*S. californicus*			
Organs of plants	Fe	Cu	Zn	Pb	Fe	Cu	Zn	Pb
Roots	6.26 ± 6.28	0.72 ± 0.94	5.22 ± 4.38	0.63 ± 0.28	28.39 ± 36.86	2.32 ± 3.24	27.34 ± 38.35	0.62 ± 0.43
Stems	3.24 ± 1.43	0.05 ± 0.05	1.53 ± 0.27	0.70 ± 0.46	8.08 ± 2.58	0.31 ± 0.17	4.28 ± 2.79	1.69 ± 0.01

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F-Value	p-Value	Sum of squares	Degrees of freedom	Mean squares	F-Value	p-Value
Organs of plants	66.795	1	6.680	4.076	0.137	245.422	1	245.422	3.211	0.171
Metal cation	269.968	3	8.999	5.492	0.098	504.397	3	168.132	2.200	0.267
Within (Error)	49.156	3	1.639			229.300	3	76.433		
Total	385.920	7				979.119	7			

Table 3. Heavy Metal Concentrations (μg g^{-1}) in *P. australis* and *S. californicus* Determined by FAAS after Digestion Using Wet Digestion Method

	P. australis				*S. californicus*			
Organs of plants	Fe	Cu	Zn	Pb	Fe	Cu	Zn	Pb
Roots	28.10 ± 16.94	2.44 ± 1.66	2.36 ± 0.93	0.56 ± 0.28	107.91 ± 3.01	5.14 ± 0.30	4.38 ± 2.41	1.42 ± 0.19
Stems	4.08 ± 3.85	0.22 ± 0.06	1.08 ± 0.69	0.29 ± 0.05	13.51 ± 1.47	0.39 ± 0.03	1.38 ± 0.00	1.59 ± 0.26

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F-Value	p-Value	Sum of squares	Degrees of freedom	Mean squares	F-Value	p-Value
Organs of plants	96.536	1	96.536	1.483	0.310	1299.99	1	1299.99	1.230	0.348
Metal cation	336.197	3	112.066	1.722	0.333	5105.33	3	1701.78	1.610	0.353
Within (Error)	195.265	3	65.088			3171.49	3	1057.16		
Total	627.998	7				9576.80	7			

concentrations determined in the roots and stems of both plants. The ANOVA analysis to determine if there is an influence of the organ of each plant or the nature of the metal ion in its concentration is also included in both tables. Figure 2 shows the comparison of the average concentrations of heavy metals in both plants.

Table 2 and Table 3 present the heavy metal concentrations determined in the roots and stems of both plants. The highest level of Fe, Cu, and Zn were found as 107.91 μg g^{-1}, 5.14 μg g^{-1}, and 27.34 μg g^{-1} in the roots of *S. californicus* respectively. The concentration of Fe (6.26 μg g^{-1}) in the roots of P. australis, determined by the DA method, is very similar to that reported by Prica *et al*. (6.79 μg g^{-1}) [78].

Very close values for Cu removal were reported by Murray-Gulde *et al*. [22], whereas the lowest level for those metals was 3.24 μg g^{-1}, 0.05 μg g^{-1}, and 1.08 μg g^{-1} in the stems of *P. australis* respectively. We can notice that the concentration of Cu in roots obtained using the WD method was 5.14 μg g^{-1}, a value close to 5.64 μg g^{-1} reported in an experimental text using the *S. californicus* [22]. The Cu value of 2.44 μg g^{-1} determined in the roots of *P. australis* using the WD method (Table 3) was very similar to the 2.6 μg g^{-1} and 2.7 μg g^{-1} reported by Peverly *et al.* [79] and St-Cyr *et al.* [80], respectively. On the other hand, the value of 2.31 μg g^{-1} reported by Bonanno *et al*. [23] is almost the same as that determined in the present experiment using the DA method in *S. californicus* (Table 2).

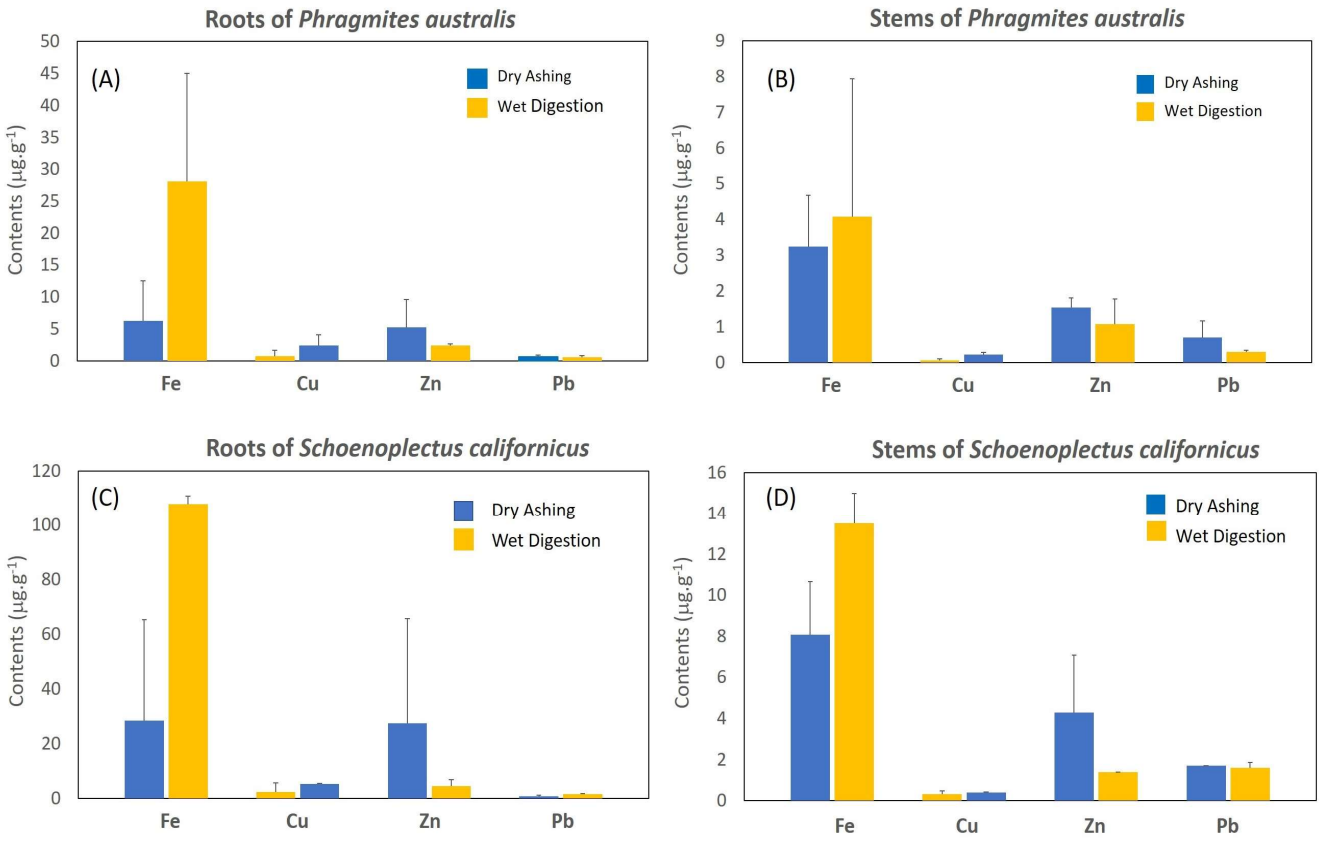

Fig. 2. Metal concentrations obtained by Dry Ashing and Wet Digestion in P. australis a) roots and b) stems; and in S. californicus c) roots and d) stems. Columns indicate means (± one standard error) of two replicates.

The concentration of Zn (5.22 µg g^{-1}), in the roots of P. australis, determined by the DA method, is very close to 6.8 µg g^{-1} reported by Bragato *et al*. [81] using the same plant. On the other hand, the concentration of 27.34 µg g^{-1} of Zn determined by the DA method in the roots of *S. californicus* is within the range of concentrations 20-80 µg g^{-1} previously reported by Bragato *et al*. [82] using *P. australis.*

Regarding Pb, its concentration of 0.56 µg g^{-1} determined in the roots of P. australis with the WD method is very close to that of 0.43 µg g^{-1} reported by Zhang *et al.* [83] using the same plant. The lowest and highest contents of Pb were found as 0.29 µg g^{-1} in stems of *P. australis*, and 1.69 µg g^{-1} in stems of *S. californicus*, respectively. In addition, the roots/stems ratio for Pb obtained using the WD method was 1.93 in *P. australis*. A similar value of 1.68 was previously reported [23].

In Table 4 it can be compared that the methods are selective for each metal. This is because, during the phytoremediation process, metals can form organic or inorganic compounds inside the plant [80,84]. In the DA method, water and other volatile materials present in the sample are vaporized upon heating and the organic matter present in the sample is burned in the presence of oxygen in the air. Also, most of the minerals present in the sample are converted into sulfates, nitrates, phosphates, chlorides, and silicates [85]. While the WD method involves heating in the presence of strong acids and oxidizing agents [86]. And, the heating needs to be carried out until the organic matter is completely decomposed. Thus, this leaves only mineral oxides in the solution. From the results obtained, it can be inferred that Zn and Pb are forming more inorganic than organic compounds. In fact, Cu and Fe can form organic complexes with phytochelatin (PC), which is synthesized by the plant when it is in an environment with these elements

Table 4. Mass Balance Calculations of Heavy Metals in *P. australis* and *S. californicus* (10 days)

Metal	Plant	Initial concentration (μg)	Final concentration (μg)	DA		WD	
				Net accumulation in plant (μg)	R (μg)	Net accumulation in plant (μg)	R (μg)
Fe	Phr	216 ± 2.0	13.5 ± 1.5	17.2 ± 1.8	185.3 ± 5.0	52.97 ± 1.8	149.53 ± 2.5
	Sch	216 ± 2.0	35.5 ± 1.0	75.9 ± 2.2	104.59 ± 3.8	125.9 ± 3.6	54.60 ± 1.0
Cu	Phr	243 ± 4.1	6.5 ± 2.2	1.8 ± 0.5	234.74 ± 6.5	4.38 ± 0.8	232.12 ± 3.7
	Sch	243 ± 4.1	3.5 ± 2.0	6.0 ± 0.2	233.46 ± 4.8	5.86 ± 0.5	233.64 ± 4.2
Zn	Phr	241 ± 3.2	4.0 ± 1.0	11.4 ± 1.5	225.6 ± 3.5	5.06 ± 1.2	231.94 ± 2.8
	Sch	241 ± 3.2	32.0 ± 2.2	72.7 ± 4.0	136.29 ± 2.2	8.17 ± 1.3	200.83 ± 1.9
Pb	Phr	224 ± 1.5	7.0 ± 1.2	2.1 ± 0.5	214.93 ± 1.8	1.18 ± 0.8	215.82 ± 3.2
	Sch	224 ± 1.5	2.5 ± 1.0	2.8 ± 0.5	218.75 ± 1.7	3.46 ± 0.2	218.04 ± 2.8

Phr = *Phragmites australis*; Sch = *Schoenoplectus californicus*.

[87-89]. Cu-PC and Fe-PC complexes may partially volatilize in the first part of the DA method. For this reason, the concentrations of Cu and Fe are lower with the DA method than with the WD method. Therefore, the WD method must be used because the organic complexes of Cu and Fe are lost in the incineration step of the DA method. In addition, the Cu and Fe elements can be incorporated into a silicate, causing them to remain in the insoluble residue when the usual digestion reagent, HNO_3, was used (second part of the DA method).

In the case of Zn, its complex with PC is thermodynamically less stable than the Cu-PC [90]. So, there is competition in the formation of the M-PC complex with Cu. In this sense, since Zn does not preferentially form Zn-PC complexes, there will not be a great loss of this element during calcination. The DA method ensure total destruction of the organic matter; then the associated elements are generally transformed into carbonate or oxide forms. ZnS is insoluble in HNO_3. During the calcination process, ZnS can form $ZnSO_4$ which is stable up to 680 °C [91]. Since $ZnSO_4$ is soluble in HNO_3, Zn does not remain in the insoluble residue after adding HNO_3 to calcine [92]. So, there will be no loss of Zn by the DA method. In the case of Pb, $Pb(NO_3)_2$ can be thermally decomposed to PbO at temperatures between 200-470 °C. $Pb(NO_3)_2$ is insoluble in HNO_3, while PbO is [93]. This would explain why Zn and Pb can be optimally recovered by the DA method than the WD method. Similar results were reported by Gong *et al.* [94].

In addition, the determination of the concentration of an element in an organic matrix would probably involve several aspects such as sampling, disruption of the sample, manipulation, and measurement. Each one can give rise to errors that will affect the accuracy of the final result [85]. Likewise, the conditions of each treatment method can affect the result. In fact, the determination of metal concentrations in plant matter using the previous DA method can be affected by some experimental parameters such as temperature, duration, and container material. Volatilization of some components could occur at temperatures above 500 °C. Likewise, an inappropriate temperature and duration in the calcination can imply incomplete combustion, affecting the final results [95]. Finally, porcelain and silica containers used in the DA method can alter the metal content of the recovered ash. Platinum may be satisfactory to avoid contamination. A suitable DA procedure might be established for any specific plant matter but the exact conditions would have to be carefully determined. In the WD method, the factors that can influence the results are the type of reagent, its concentration, the mixture of reagents, the ratio of sample weight to reagents, and digestion time. All of these factors may not dissolve the entire sample, since some oxides and silicates may have poor solubility. In addition, the reagent used depends on the nature of the matrix. The final results can be affected by many others sources of potential errors, *i.e.*,

partial digestion of the analytes present, or some type of contamination from the vessels of chemical products used. WD procedure is more rapid and is much less subject to contamination from outside sources of metals. This advantage is a very important one. However, the WD method is limited by a low maximum digestion temperature, which cannot exceed the ambient-pressure boiling point of the corresponding acid or acid mixture [96].

Likewise, a mass balance was carried out in order to determine the accumulation of Fe, Cu, Zn, and Pb in the roots and branches of both plants using the formula suggested by Kumari *et al.* [17]:

$$NA\ (mg) = \left(C_i - C_f - R\right)\ x\ V \quad (3)$$

Where NA is the net accumulation of heavy metals in the harvested plants, C_i and C_f are the average concentrations of heavy metals in the wastewater before and after treatment, respectively, R is the mass of heavy metals lost by precipitation in the substrate, V = is the volume of residual water used in the experiment. The results are presented in Table 4.

Mass balance calculations revealed that the loss of Fe, Cu, Zn, and Pb by natural precipitation was higher than its net accumulation in plant tissues in both plants. Being only higher for Fe in *S. californicus* Kumari [17] has reported that natural precipitation occurs through the oxidation of metal ions in the presence of oxygen [97-102]. In this case, the metal ions may have formed oxidized salts in the substrate of the experimental wetland.

Indistinctly, if the DA or WD method is used, it is observed that the tendency of net accumulation of heavy metals in the tissues of both plants is Fe > Zn > Cu > Pb. A similar trend was reported by Kumari et al. when using *P. australis* [17]. On the other hand, it is evident that the Net Accumulation is higher when *S. californicus* is used compared to *P. australis*.

P. australis is one of the most extensively studied aquatic plants for heavy metal removal. In fact, this plant has a high potential for metal removal as well as the possibility of accumulating them in both aerial and underground biomass [103,104]. It has been shown that the roots of *P. australis* can accumulate high concentrations of Mn, Fe, and Cu [105].

It has been established that the behavior of metals inside the plant and therefore their toxicity does not depend only on the total concentrations of metals, but also depend on the type of plants and the mechanisms involved in the sequestration and translocation of metals inside the plant [78]. Then, *P. australis* and *S. californicus* can assimilate heavy metals, immobilizing them in their tissues without being affected. This is possible up to a certain maximum limit of toxicity. A previous study determined the toxic concentrations for *P. australis* in 500 μg g^{-1} Fe, 20 μg g^{-1} Cu, 100 μg g^{-1} Zn and 20 μg g^{-1} Pb [106]. *P. australis* tends to release an excess of metal ions through a process of transpiration, which allows it to reduce toxic concentrations in the tissues of its leaves [107]. Exceeding these toxicity limits, plants will use mechanisms to eliminate heavy metals and thus prolong their life. Thus, *P. australis* can regenerate to a certain extent, as long as the maximum toxicity limits of the plant are not exceeded. On the other hand, *P. australis* is a fast-growing plant [108]. In addition, this plant has a high capacity for acclimatization to environmental conditions considered adverse [109].

The concentrations of Fe, Cu, Zn, and Pb in *P. australis* determined by both methods in roots and steams, are below the toxic concentrations reported by Kalra *et al.* [94]. In this sense, it can be concluded that the plant could regenerate without any inconvenience.

In addition, a two-way ANOVA was used to compare the mean of metal concentration obtained using both digestion methods. The steps to ANOVA were: 1) Formulate the hypothesis, 2) Set a significance level (α), 3) Compute an F-Statistic, 4) Use the F-Statistic to derive a p-value, and 5) Compare the p-value and significance level to decide whether or not to reject the null hypothesis.

The F-statistic is simply a ratio of the variance between sample means to the variance within sample means [110]. The variance between is the ratio of the sum of squares between groups to degrees of freedom between groups, simply the number of groups minus 1. While variance within the sample means the ratio of the sum of squares within groups to degrees of freedom within groups, simply the number of data points minus the number of groups [111].

F critical value is the value found in the F-distribution table [112] with n_1-1 and n_2-1 degrees of freedom and a significance level of α, F (α; n_1-1; n_2-1). For organs of both plants, n_1-1 = 1, n_2-1 = 3 and α = 0.05, (F (0.05; 1; 3)); while for the metal cation specie, n_1-1 = 3, n_2-1 = 7 and α = 0.05,

(F (0.05; 3; 7)).

Then, the results of ANOVA with a 95% confidence level for organs of both plants (*P. australis* and *S. californicus*) using both digestion methods (DA and WD) were (F (0.05; 1; 3 = 10.128)); and for the metal, cation specie were (F (0.05; 3; 7) = 9.277)) [112]. Results of Table 2 and Table 3 show that the F critical value for organs of *P. australis* samples treated using DA and WD are greater than the calculated F value (10.128 > 4.076 and 10.128 > 3.211). Similar results were observed for *S. californicus* where the F critical value for organs of plants is greater than the calculated F value (10.128 > 1.483 and 10.128 > 1.230). Then the organ of each plant does not influence the results.

The ANOVA results for the nature of the metal ions show that the F critical value of *P. australis* samples is greater than the calculated F value (9.277 > 5.492) and (9.277 > 2.200) when samples were treated using DA and WD method respectively (Table 2). Similar results were observed for *S. californicus* where the F critical value is greater than the calculated F value (9.277 > 1.722 and 9.277 > 1.610) (Table 3). Therefore, the heavy metal cation does not influence the results.

The results of the Student's t-test are presented in Table 5. Using Eqs. (1) and (2), the values of t_{exp} and RSD were determined for each element in an organ of *P. australis* and *S. californicus* (Table 5). The $t_{theoretical}$ is obtained through t distribution tables, considering the level of confidence and the degrees of freedom ($\alpha < 0.05$; n_1+n_2-1). If the $t_{exp} < t_{theoretical}$, then the null hypothesis (h_0 = there is no significant difference in both methods used) is accepted; Otherwise, if there is a difference between the two methods used in the analysis of metal concentration in plant tissues. Table 5 shows that in the roots of *P. australis* there was no significant difference for Fe (1.33 < 3.182), Cu (0.94 < 3.182), Zn (1.17 < 3.182) and Pb (0.99 < 3.182) since the t_{exp} is less than the $t_{theoretical}$. The same trend is observed for the leaves of *P. australis* for all metals,

Table 5. Summary of t-values and Mean Values of Concentration of Heavy Metals (μg g^{-1}) from Dry and Wed Digestion Method

P. australis									
	Method	Fe		Cu		Zn		Pb	
		$\bar{x}$	RSD	$\bar{x}$	RSD	$\bar{x}$	RSD	$\bar{x}$	RSD
Roots	WD	28.10	0.43	2.44	0.48	2.36	0.28	0.56	0.36
	DA	6.26	0.71	0.72	0.93	5.22	0.59	0.63	0.32
	t_{exp}	1.33		0.94		1.17		0.99	
Steams	WD	4.08	0.67	0.22	0.18	1.08	0.45	0.29	0.12
	DA	3.24	0.31	0.05	0.64	1.53	0.13	0.70	0.47
	t_{exp}	1.13		2.91		3.67		2.17	
S. californicus									
	Method	Fe		Cu		Zn		Pb	
		$\bar{x}$	RSD	$\bar{x}$	RSD	$\bar{x}$	RSD	$\bar{x}$	RSD
Roots	WD	107.91	0.02	5.14	0.04	4.38	0.39	1.42	0.10
	DA	28.39	0.92	2.32	0.99	27.34	0.99	0.62	0.50
	t_{exp}	3.32		1.36		0.80		1.82	
Steams	WD	13.51	0.08	0.39	0.06	1.38	0.01	1.59	0.12
	DA	8.08	0.23	0.31	0.39	4.28	0.46	1.69	0.01
	t_{exp}	6.90		0.55		1.47		0.56	

$\bar{x}$ = mean values of concentration of heavy metals (μg g^{-1}).

except for Zn where the t_{exp} is greater than the $t_{theoretical}$ (3.67 > 3.182). Then DA method is more suitable for determining Zn in steams of *P. australis* than the WD one due to obtaining less RSD% (0.13 < 0.45). In the roots and stems of *S. californicus* there is no significant difference between the two methods to determine Cu (1.36 < 3.182 and 0.55 < 3.182), Zn (0.80 < 3.182 and 1.47 < 3.182) and Pb (1.82 < 3.182 and 0.56 < 3.182) since the values of t_{exp} is lower than the $t_{theoretical}$ determined in tables. In the case of the determination of Fe both in roots and stems of *S. californicus*, the t_{exp} is greater than the $t_{theoretical}$ (3.32 > 3.182 and 6.90 > 3.182), so there is a difference in the application of both methods. Then WD method is more suitable for determining Fe in *S. californicus* than the DA method due to obtaining less RSD% (0.02 < 0.92 and 0.08 < 0.23).

Finally, if we compare both methods to determine Cu and Pb in *P. australis* and *S. Californicus* using the Student's t-test, it is observed that there is no significant difference (Table 5). In addition, WD and DA data for Fe in *S. californicus* show significantly different results based on $P < 0.05$. Taking into account these results, it is observed that the WD method is more sensitive than the DA one.

Figure 2 shows the comparison of the average concentrations of heavy metals in both plants. The differences between the means of Cu and Pb elements were not significant; whereas the differences between means of Fe and Zn elements were more evident. In the case of Fe concentration from both roots of *P. australis* and stems of *S. californicus*, the WD method provides higher values. Likewise, for Zn, it is observed that there is a greater difference between the DA and WD method for both the roots and the stems in *S. californicus*.

CONCLUSIONS

Based on the analysis of the results obtained by the two methods, it can be concluded that the highest concentrations of copper, iron, and zinc are found in the roots of *P. australis* and *S. californicus* using both the DA and the WD method. For lead, a similar behavior is observed only in the root samples of *S. californicus* treated with the DA method. While for the stems it is better to use the WD method. From the results obtained and the statistical evaluation of the data, the WD method gives better results than the DA method. The observed differences are due to different compounds formed for each metal ion inside the plant tissue. A random error could be also included.

Copper is preferably removed by the *S. californicus* while the *P. australis* preferably removes zinc. It is concluded that both *P. australis* and *S. californicus* can be used to remove heavy metals from water, and restore and reclaim abandoned mining sites through mine closure plan projects. The tendency of net accumulation of heavy metals in the tissues of both plants is Fe > Zn > Cu > Pb indistinctly if the DA or WD method is applied. In addition, a pilot experiment with a dynamic flow must be carried out.

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