Determination of mercury in vegetal tissues by microPIXE: Application to the study of hyperaccumulation by *Spirodela intermedia* (Lemnaceae)

Determinación del mercurio en tejidos vegetales por microPIXE: Aplicación al estudio de la hiperacumulación por *Spirodela intermedia* (Lemnaceae)

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Summary

Background and aims: Aqueous mercury (II), Hg^{2+}, is still nowadays a hazardous pollutant with a large dispersion. Phytoremediation strategies are an environmental friendly and low-cost alternative. In order to improve these processes, *Spirodela intermedia*, an autochthonous floating macrophyte, was used to remove Hg^{2+} from mineral water under laboratory conditions, studying the *in vivo* distribution of mercury and other elements by nuclear microprobe scanning mapping.

M&M: Exposures (1 and 10 $mg.L^{-1}$ Hg^{2+} concentrations) were performed during at least 2 weeks. All the parameters from the bioremediation process as uptake rate, bioconcentration factors (BCFs) of mercury in roots and leaves and translocation factors (TFs), were achieved from microPIXE quantifications at Buenos Aires Tandar accelerator.

Results: For 1 and 10 $mg.L^{-1}$ concentrations, *S. intermedia* can be considered as a hyperaccumulator. The highest BCFs (> 1000 in roots and > 200 in leaves) were obtained for 1 $mg.L^{-1}$ of Hg^{2+} at 96 h. In all cases TFs < 1 were measured, indicating that Hg^{2+} translocation is not taking place. High resolution spatial 2D maps of the *in vivo* distribution for different exposure conditions were established. It was observed that Hg^{2+} distribution in leaves is more heterogeneous than in roots. An important finding was the detection of Hg in chlorenchyma where its effects are more toxic. Correlation between mercury and calcium distribution and its relationship with physiological responses to intoxication have been examined.

Conclusions: Phytoremediation of Hg^{2+} by *S. intermedia* is a convenient alternative. Since the protocol was performed using a real water, it becomes an advisable tool at higher scale.

Key words

Spirodela intermedia, mercury, hyperaccumulation, microPIXE.

Resumen

Introducción y objetivos: El mercurio (II) acuoso, Hg^{2+}, es todavía un contaminante peligroso ampliamente distribuido. Las estrategias de fitorremediación son ambientalmente amigables y de bajo costo. Con el fin de optimizar estos procesos, se utilizó *Spirodela intermedia,* una macrófita acuática autóctona, para remover Hg^{2+} en agua mineral, en condiciones de laboratorio, estudiando la distribución *in vivo* de mercurio y otros elementos por mapeo barriendo con una microsonda nuclear.

M&M: Las exposiciones (concentraciones de Hg^{2+} de 1 y 10 $mg.L^{-1}$) duraron al menos 2 semanas. Todos los parámetros del proceso como tasa de captación, factores de bioconcentración (BCFs) de mercurio en raíces y frondes y factores de translocación (TFs) fueron calculados a partir de cuantificaciones de microPIXE con el acelerador Tandar de Buenos Aires.

Resultados: *S. intermedia* puede ser considerado un hiperacumulador. Los más altos BCFs (> 1000 en raíces y > 200 en frondes) correspondieron a 1 $mg.L^{-1}$ a las 96 hs. En todos los casos, se constató que TFs < 1, indicando que no ocurre translocación de Hg^{2+}. Se obtuvieron mapas 2D de alta resolución espacial de la distribución elemental *in vivo* para las diferentes condiciones. Se observó que la distribución de mercurio en frondes es más heterogénea que en raíces. Fue importante la detección de Hg en clorénquima donde sus efectos son más tóxicos. Se analizó una correlación entre la distribución de mercurio y calcio y la relación con respuestas fisiológicas.

Conclusiones: La fitorremediación de Hg^{2+} con *S. intermedia* es una alternativa conveniente. Por haberse realizado en agua real, el protocolo es escalable.

Palabras clave

Spirodela intermedia, mercurio, hiperacumulación, microPIXE.

Introduction

The impact of mercury compounds in the natural environment represents even now a very important matter (Nriagu, 1979). 1 $\mu g.L^{-1}$ has been established as maximum level for human water consumption in Argentina (Código Alimentario Argentino, 2012). Particularly, in Argentina, mercury compounds were employed as pesticides in tobacco plantations (García *et al.*, 2003). In last years, the presence of Hg contamination in groundwater and surface water has been a very topical issue due to the spillage of cyanide solutions produced by the activity of the Veladero mine in the province of San Juan, Argentina (Ford *et al.*, 2015). Methylmercury, the most toxic mercury species, is produced by biotic and abiotic Hg^{2+} methylation (Celo *et al.*, 2006; King *et al.*, 2001; Yin *et al.*, 2012). To avoid this serious process, aqueous Hg^{2+} must be treated.

The treatment of aqueous mercury is problematic. Chemical procedures present several drawbacks, such as solid wastes disposal (Serpone *et al.*, 1988). More recent removal strategies, known as advanced oxidation technologies (AOT's), improve highly the efficiency of inorganic and organomercurial compounds removal. AOT experiments are generally performed in pure water (de la Fournière *et al.*, 2007 and references therein) but, in real waters, removal rates are remarkably lower.

Bioremediation is more environmentally friendly and is largely cheaper than chemical treatments. Phytoremediation by aquatic macrophytes has been especially widely reported motivated by their hyperaccumulating capacities of the soluble and bioavailable contaminants, chiefly metals and metalloids, from water (Miretzky *et al.*, 2004; Mishra *et al.*, 2009; Rahman & Hasegawa, 2011). Floating macrophytes accumulate mainly in their roots (Vardanyan & Ingole, 2006). Autochthonous organisms should be selected to avoid any ecosystem unbalance. Consequently, in this work, remediation experiments using duckweed *Spirodela intermedia W. Koch* (Lemnaceae) have been carried out. This species is reported in lentic water bodies of Argentina and other regions of Central and South America (Feijoo & Lombardo, 2007; Basílico *et al.*, 2013). In this paper, removal of $HgCl_2$ dissolved in mineral water is studied, focusing on the *in vivo* mercury distribution. Absorption process modeling and correlation between mercury and calcium uptake, possibly involved in a mechanism of resistance, have been also investigated.

Material and methods

In this study, the spatial distribution of Hg was analyzed by microPIXE scanning mapping, namely PIXE with highly-focused ion beams with micrometric dimensions (Barnabas *et al.*, 1999). The sensitivity of this technique allows simultaneous mapping of main, minor and trace elements. Combined with STIM (Scanning Transmission Ion Microscopy) and micro-RBS (Rutherford Back Scattering) provides the quantitative determination of trace elements concentration with high sensitivity ($\mu g.g^{-1}$ range) and multi-elemental distribution maps with high spatial resolution (micrometric range) of the irradiated region conserving the structure of the sample (Lefevre *et al.*, 1991; Witkowski *et al.*, 1997, Barnabas *et al.*, 1999). Usually, the typical detection limits for most elements are in the range of 1−10 $\mu g.g^{-1}$.

The heavy ion beam microprobe in Buenos Aires

Focusing is performed with the aid of the Tandar Laboratory heavy-ion microprobe onto a spot of about 3-5 μm in diameter on Hg contaminated transversal sections of roots and aerial parts and of non-contaminated controls. The samples were irradiated normal to the incident particle beam and measured at 135° with respect to the beam direction, to minimize the X-ray background. The largest area that can be scanned under these conditions is about 1×1 mm^2 which it is large enough to hold several cross sections roots of *Spirodela intermedia*. The X-rays belonging from the sample were measured with a 80 mm^2 high resolution silicon drift detector X-ray detector (http://www.ketek.net/). A sheet of Kapton 50 μm thick was placed in front the detector to shield it against backscattered ions and to attenuate X-rays from the light elements minimizing the pile-up in the X-ray spectrum. This sheet has not effect on the transmission at the energy of the mercury L_α line. More details of the experimental setup can be found in reference (Stoliar *et al.*, 2004).

Sample collection and preparation

S. intermedia were collected from a natural

wetland, carefully washed with Milli-Q water and placed in glass bottles filled (6 plants per bottle) illuminated with fluorescent light (Basílico *et al.*, 2013) in a 16:8 h (light:dark) photoperiod. 250 mL of commercial mineral water was employed, spiked or not with Hg^{2+}, without any nutrient addition since we were focused on groundwaters remediation. Characterization of the mineral water is shown in Table 1. Initial concentration of Hg^{2+} was 1 and 10 $mg.L^{-1}$. Exposure time was from hours to weeks (12, 24, 48, 72, 96, 168 and 336 h).

In order to analyse mercury content in roots and leaves, at least three plants of each condition were withdrawn and immediately frozen. Transversal cross-sections ~ 10 µm thick of roots (Fig. 1) and leaves were obtained using a cryo-microtome at -20 °C to avoid ions migration which could alter *in vivo* distribution. Next, histological cross-sections were transferred to ultrapure polypropylene backings with acrylic-glass target frames and freeze-dried (with no further processing) and finally irradiated with the microbeam (Llabador & Moretto, 1996).

Table 1. Chemical characterization of mineral water used.

pH	EC	Ca^{2+}	Mg^{2+}	Na^{+}	F^{-}	K^{+}	Cl^{-}	HCO_3^{-}	SO_4^{2-}	TDS
	$mS.cm^{-1}$	$mg.L^{-1}$	$mg.L^{-1}$	$mg.L^{-1}$	$mg.L^{-1}$	$mg.L^{-1}$	$mg.L^{-1}$	$mg.L^{-1}$	$mg.L^{-1}$	$mg.L^{-1}$
8.1±0.2	230±21	30±2	3.0±0.2	10±0.6	1.14±0.36	4±0.4	4±0.3	79±5	44±3	176±12

Abbreviations: EC: Electrical conductivity. DS: Total dissolved solids.

For each section, a ‘‘twin’’ contiguous section was stained to obtain an ‘‘as-close-as-possible’’ optical image of the freeze-dried section, to distinguish details of the histology which are not readily observable in the irradiated non-stained section, and correlate them with the distribution of the different elements.

Chemicals

Stock solutions were prepared using $HgCl_2$ (Merck, Darmstadt, Germany) of the highest purity with Milli-Q water (resistivity = 18 MΩ.cm). As sample mounting was used a thin 4 µm thick Prolene film (Fluxana GmbH & Co., Bedburg-Hau, Germany, www.fluxana.com). This film was selected because microPIXE analysis of the Prolene gave a remarkably clean spectrum, showing that it contains no contaminants above the minimum detectable limit (MDL) (Southworth-Daviesa *et al*., 2007). The polypropylene was mounted on an aluminium support with a 10 mm diameter hole, which is held in a “holder-ladder” capable of holding up to three samples, inside the vacuum chamber.

MicroPIXE analysis

The aim this work was to investigate the distribution of Hg in roots cross-sections of the floating macrophyte *Spirodela intermedia* by microPIXE (micro-Particle Induced X-ray Emission) spectrometry (Mesjasz-Przybyłowicz & Przybyłowicz, 2002; Lyubenova *et al*., 2007; Vogel-Mikuš *et al.*, 2007; Cestone *et al*., 2012; Wang *et al*., 2013; Módenes *et al.*, 2013) to permit a better understanding of the uptake, localization, accumulation and translocation of the metal in this

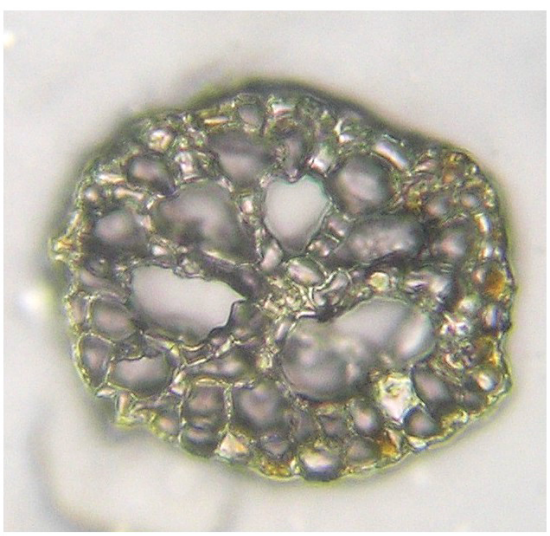

Fig. 1. Typical root cross-section OM photograph of *S. intermedia*. Scale= 20 µm.

plant, as well as to know and quantify the ability of this particular plant species to hyperaccumulate and tolerate the concentration of metals in their roots. This knowledge is critical to examine phytoextraction strategies through phytoaccumulation in aquatic contaminated areas.

There are several analytical tools ranging from those which allow quantify from "bulk" analysis the concentrations of metals in plants to those that have the ability to quantify and expose the elemental distribution of metals in plant tissues (IAEA, 1980; Lefevre *et al.*, 1991; Witkowski *et al.*, 1997; Barnabas *et al.*, 1999; Malan *et al*., 2012; Mendes Godinho *et al.*, 2013).

Of the various nuclear analytical methods using ion beams, PIXE is the more often used technique. PIXE is a high sensitivity, multi-elemental analysis technique, based on the high cross-section MeV-ion ionization of inner-shell vacancies (mainly in the K-and L-shell) and detection of subsequent emission of characteristics X-rays which yields information on the concentration of the elements present in the samples (Lefevre *et al*, 1991; Witkowski *et al.*, 1997).

Thin samples of 10 µm thickness are easily broken during handling and only cuts which kept cell integrity under test by optical microscope, were irradiated. The samples were mounted on a manual xyz translator (2 µm resolution step) in the irradiation chamber. The rough positioning of the samples was achieved using an optical digital microscopy.

The samples were irradiated with a 50 MeV energy $^{16}O^{5+}$ ion beam focused to a < 5 µm spot size scanning the beam over a 256×256 pixel matrix. Data acquisition was carried out by scanning the beam over a given sample and saving the data at each pixel along with the simultaneous X and Y coordinates of the beam spot. These matrix data (X-ray energy, X-Y coordinates) allow us to obtain the full energy X-ray spectrum and the Hg and other elements distributions by windowing on interest X-ray lines in the PIXE spectrum. Fig. 2 shows such a spectrum for a root tissue section.

The dry mass was estimated using STIM with a $^{16}O^{5+}$ ion beam at 50 MeV with an intensity of about 10^3 ions per second. Since the samples are completely traversed by the ^{16}O beam, it is possible to determine the effective density of the sample using the energy loss contrast STIM method (Lefevre *et al.*, 1987). Considering that the dried plant tissue samples were sliced on the cryo-microtome to a thickness of 10±0.5 µm, the STIM measurement gives 0.36±0.09 g.cm^{-3} as equivalent bulk tissue density. This great uncertainty

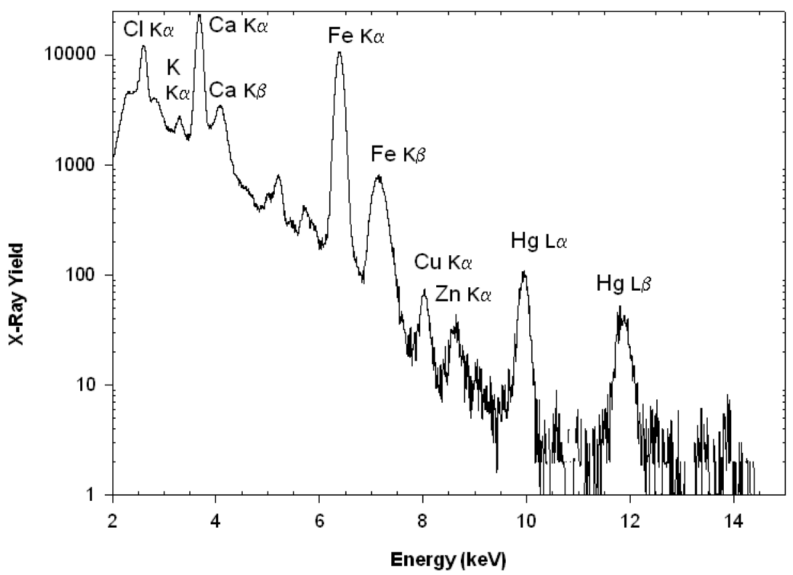

Fig. 2. MicroPIXE spectrum of an irradiated root cross-section of *Spirodela intermedia* exposed to 10 mg.L^{-1} Hg^{2+} solution during two weeks.

in the result is intrinsic to the STIM method since the heterogeneous morphology of the sample results in an uneven area density after freeze-drying (Vogel-Mikuš *et al.*, 2009). This value doubles the mean root tissue density ~0.15 g.cm^{-3} determined from morphological parameters of floating macrophyte *Lemna minor* (Cedergreen & Vinbæk Madsen, 2002). However, this result is acceptable if one considers the great variability of the root density determined for different plant species. Birouste *et al.* (2014) measured root density variations between 0.152 and 0.683 g.cm^{-3} using the Archimedes' method. With this equivalent tissue density the Hg content determined by microPIXE was normalized to express its concentration in term of µg of Hg per g of dry sample.

Data analysis

The resulting spectra were analysed off-line with the OMDAQ-2007 computer code by calculating the peak area using the Gaussian fit or directly the sum of the counts per channel under the peak. This is possible due to the cleaning of the spectrum above 9 keV (almost free background region - excellent peak-to-background ratio) where only the X-ray peaks of the Hg lines L_α and L_β are present and are fully resolved. By gating on the Hg L_α line, we constructed a two-dimensional Hg map which shows where and how the mercury penetrated and distributed in the transversal section of the roots.

Likewise two-dimensional maps of elemental concentration for any other element present in the X-ray spectrum can be obtained by gating the corresponding X-ray lines.

The *in vivo* microPIXE distribution maps of Hg (gate on Hg L lines), K and Ca in the roots of *S. intermedia* are shown in Fig. 3. The microPIXE images of the major elements K and Ca were chosen to indicate the structure of the root cross-section. The distribution of mercury in roots (Fig. 3) after 24 hours exposure to Hg^{2+} (10 mg.L^{-1}) seems to be quite homogeneous during the uptake process if compared with *in vivo* distribution of mercury obtained by Lomonte *et al.* (2014) in roots of *Chrysopogon zizanioide*.

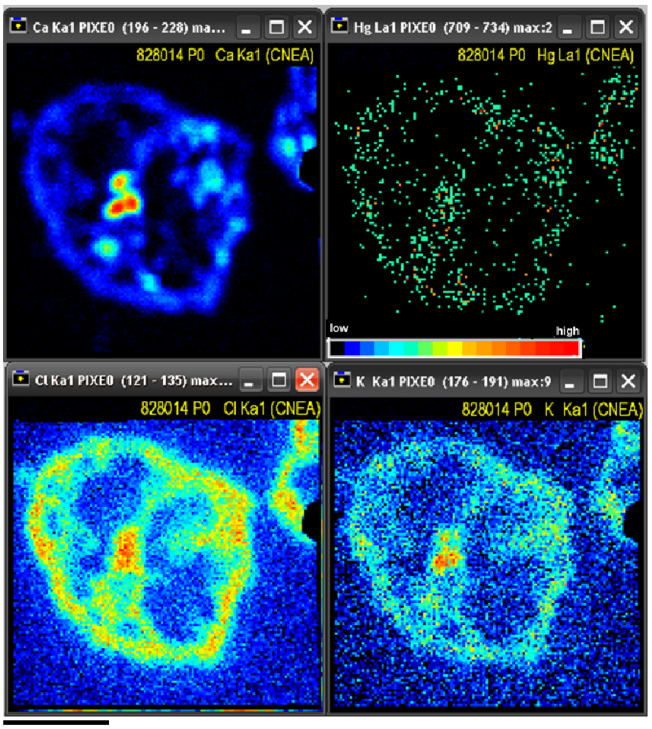

Fig. 3. 2D X-rays maps corresponding to Ca, Hg, Cl and K distribution from the root cross-section of *S. intermedia* exposed to Hg^{2+} (10 mg.L^{-1}) during 24 hours. MicroPIXE conditions: 50 MeV $^{16}O^{5+}$ beam, scan size 200×200 µm^2, spot size 3×3 µm^2. The pixels change color from blue to red with the increase of the elemental concentration (see color scale included in the figure). Scale= 50 µm.

Results

Uptake of mercury

Fig. 4 shows the temporal evolution of the uptake of Hg^{2+} for the concentrations of 1 and 10 $mg.L^{-1}$. It can be seen that Hg^{2+} is rapidly concentrated in the roots of *S. intermedia*. When exposed to 10 $mg.L^{-1}$ of Hg^{2+} solution, in the first 12 h the plant content is about 2 $mg.g^{-1}$ (see Fig. 4). Over the next 84 hours increases nearly threefold and then drastically decreases indicating that, for this content, mercury becomes highly toxic for the plant. For an external concentration of 1 $mg.L^{-1}$, the maximum uptake of mercury is higher than that found for plants placed in a 10 $mg.L^{-1}$ medium and a saturation zone is observed from 96 h, suggesting that this mercury level is still tolerated.

Various models can be used to analyse the kinetics of sorption process. The simplest kinetic model which describes the process of sorption, is the pseudo-first order rate equation suggested by Lagergren (Yuh-Shan, 2004). This model has been most widely used for the adsorption of aqueous phase pollutants such as metal ions. Mercury net-sorption kinetics can also be modelled by this pseudo first-order equation based on solid capacity:

$$\frac{dq(t)}{dt} = v_S(t) = k.\left(q_e - q(t)\right) \quad (1)$$

where, and ($mg.g^{-1}$) are the mg of solute absorbed per g of sorbent at any time t and at equilibrium respectively, ($mg.g^{-1}.h^{-1}$) is the uptake rate, (h^{-1}) is the pseudo first-order rate constant of sorption and $k.q_e$ ($mg.g^{-1}.h^{-1}$) is the initial sorption rate. Integrating this equation for the initial conditions $q = 0$ for $t = 0$ gives:

$$q(t) = q_e.\left(1 - e^{-k.t}\right) \quad (2)$$

The kinetic parameters (Table 2) were obtained by fitting equation (2) to the experimental results. The curves predicted by this model are presented in Fig. 4. The correlation coefficient indicates that the least-squares fits are similar for both Hg concentrations.

As shown in Fig. 4, for an exposure to 10 $mg.L^{-1}$ of Hg^{2+} solution (solid line), the plant rapidly increases his concentration during the first 2 days (in just 42 hours it reaches 63% of the maximum cumulative value) and saturates to a nearly constant value of 6.2 $mg.g^{-1}$ in approximately 100 hours of

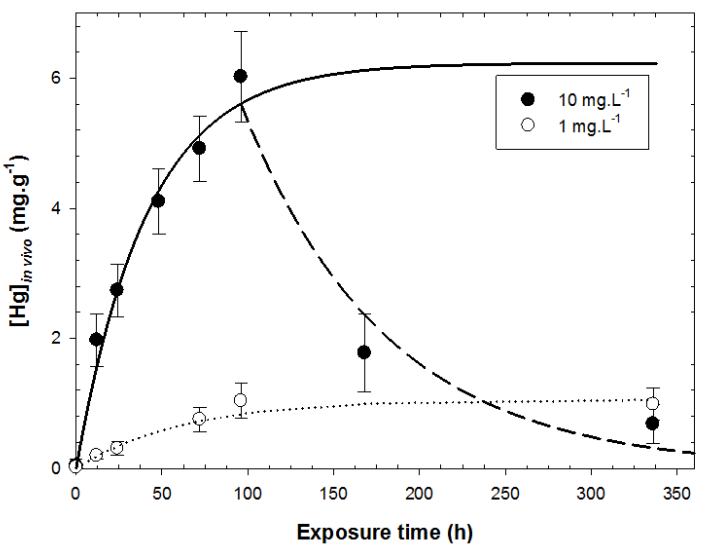

Fig. 4. Hg uptake temporal profiles in roots of *S. intermedia* exposed to aqueous Hg^{2+}. The dotted and solid lines correspond respectively to the adjustments [Eq. (2)] of the experimental values of the uptake of the plants exposed to solutions with concentrations of 1 and 10 $mg.L^{-1}$ of Hg. Dashed line indicates mercury concentration decay (for an external 10 $mg.L^{-1}$ concentration) due to plant poisoning (intoxication).

Table 2. Rate constants for the pseudo-first order equation of Lagergren fitted for removing Hg^{2+} by *Spirodela intermedia*. C_0 is the Hg^{2+} concentration in solution. S^2 is the standard deviation and R^2 the correlation coefficient of the fitting functions.

C_0 ($mg.L^{-1}$)	q_e ($mg.g^{-1}$)	$k \times 10^{-2}$ (h^{-1})	$q_e.k$ ($mg.g^{-1}.h^{-1}$)	S^2	R^2
10	6.23	2.37	0.148	0.320	0.99
1	1.06	1.6	0.017	0.122	0.97

exposure. However from that moment, it begins to release Hg to the aqueous medium due to the loss of its biological functions by poisoning (dashed line).

For a concentration of 1 $mg.L^{-1}$, the saturation zone is not followed by decay (dotted line) and the plant reaches the 63% of maximum value after approximately 63 h.

These results suggest that in remediation applications with either of both concentrations, the plants should be eliminated immediately after the fourth day. For 10 $mg.L^{-1}$ exposure, the elimination is more important because, in addition to reaching saturation, the plants start to return the captured mercury.

A possible correlation between the increase of Ca^{2+} concentration and the uptake of Hg^{2+} by roots has been evaluated. As shown in Fig. 5 and the value of the determination coefficient R^2, an exponential regression correlates quite well calcium and mercury concentrations normalized to their respective concentrations at 96 hours ($[Ca^{2+}]/[Ca^{2+}]_{96h}$).

The fitting function:

$$[Ca^{2+}]/[Ca^{2+}]_{96h}([Hg^{2+}]/[Hg^{2+}]_{96h}) = a \cdot e^{b.([Hg^{2+}]/[Hg^{2+}]_{96h})} + c \quad (3)$$

where: $a = 7.5 \times 10^{-3}$, $b = 4.5919$ and $c = 0.278$, $(a+c \approx c)$ is the Ca^{2+} value of the uncontaminated

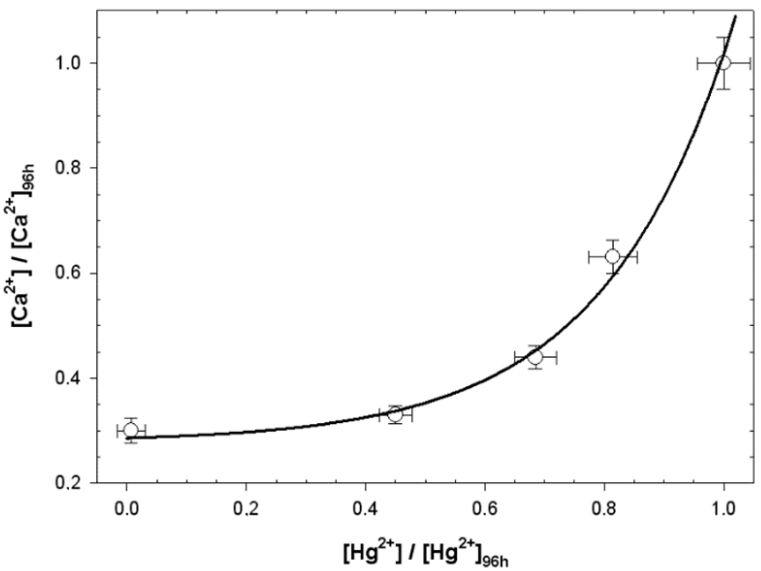

Fig. 5. Relationship between the normalized concentrations calcium $[Ca^{2+}]/[Ca^{2+}]_{96h}$ and mercury $[Hg^{2+}]/[Hg^{2+}]_{96h}$ in roots of *S. intermedia* exposed to 10 $mg.L^{-1}$ of Hg^{2+}. Time interval: 0–96 h. Solid line: regression corresponding to Eq. (3).

root (t = 0h) and b represents the growth rate of $[Ca^{2+}]/[Ca^{2+}]_{96h}$ fraction when the $[Hg^{2+}]/[Hg^{2+}]_{96h}$ normalized concentration is increasing. In accordance with this fit equation, in the analyzed samples of roots of *S. intermedia* exposed to 10 $mg.L^{-1}$ of Hg^{2+}, whenever the $[Hg^{2+}]/[Hg^{2+}]_{96h}$ increases a fraction 0.152, the Ca^{2+} uptake will increase in such way that its $[Ca^{2+}]/[Ca^{2+}]_{96h}$ shall be doubled (regardless of the initial value at t = 0 h).

A similar analysis on the concentration of 1 $mg.L^{-1}$ does not allow establishing a clear relationship between the concentrations of Ca^{2+} and Hg^{2+}.

It is reported that calcium is involved to counteract metal stress in hyperaccumulator plants (Tian *et al*., 2011). Another mechanism to avoid metal toxicity is thiol biosynthesis such as phytochelatins (PCs). In this sense, it is known that PCs are present in *Spirodela* genus (Pandey *et al*., 1999). In another hand, Ca^{2+} increases the expression of PC synthase gene under Cd^{2+} stress in *Lactuca sativa* (He *et al*., 2005). It is therefore possible that Ca^{2+}, in presence of Hg^{2+}, triggers PC synthesis in *S. intermedia*.

Translocation

Mercury in leaves has been also detected and quantified in order to evaluate possible metabolism damage during phytoremediation process. As expected, no mercury was present in leaves of controls.

Translocation factor (TF), the ratio of metal content in the leaves to those in the roots (Mattina *et al*., 2003), was calculated from experimental data:

$$TF = [Hg]_{leaves} / [Hg]_{roots} \quad (5)$$

where $[Hg]_{leaves}$ and $[Hg]_{roots}$ are the *in vivo* concentrations of mercury in leaves and roots respectively.

It is important to mention that *S. intermedia* can absorb aqueous metals through the whole plant (Porath & Pollock, 1982). Thus, in this case, it is not strictly a translocation but, anyway, TFs give valuable information when studying metabolic damage in leaves. The measured values are listed in Table 3.

In all cases, TF < 1, indicating that there is no translocation.

Bioconcentration factors in roots and leaves

From experimental and fitted data shown in Fig. 4, bioconcentration factor (BCF) of mercury in roots and leaves, defined in equations (6) and (7) was calculated for the maximum uptake reached for all the initial concentrations (96 h).

$$BCF_{roots} = [Hg]_{roots} / [Hg]_{water} \quad (6)$$
$$BCF_{leaves} = [Hg]_{leaves} / [Hg]_{water} = TF.\ BCF_{roots} \quad (7)$$

Measured values are listed in Table 4.

Table 3. Translocation factor (TF) of mercury in *S. intermedia* calculated from experimental data for different conditions.

$[Hg^{2+}]_0$ ($mg.L^{-1}$)	10	10	10	1
Time exposure (h)	48	72	96	96
TF	0.21±0.02	0.22±0.02	0.33±0.03	0.19±0.02

Table 4. Bioconcentration factors (BCF) of mercury, in roots and leaves of *S. intermedia*, calculated from experimental (mv) and fitting (fit) data, respectively.

$[Hg^{2+}]_0$($mg.L^{-1}$)	BCF_{roots}(mv)	BCF_{roots}(fit)	BCF_{leaves}(mv)
1	1294±109	1060±104	246±23
10	409±39	623±71	189±19

Discussion

As can be seen in all cases aqueous Hg^{2+} solutions, BCFs > 100 are obtained. Thus, *S. intermedia* can be considered, at least in this range of mercury concentration, as a hyperaccumulator (Rascio & Navari-Izzo, 2011). It is pertinent to mention that others methods such as membrane filtration, precipitation with chemicals, ion exchange, reduction, and adsorption are much less efficient and wasteful for concentrations lower than 100 mg L^{-1} (Manohar *et al*., 2002).

The values of BCF are in concordance with those obtained by Srivastav *et al*. (1994) using the same macrophyte for the removal of Cr ($600 \leq BCF \leq 711$) and Ni ($562 \leq BCF \leq 713$) in the range of 1−8 mg.L^{-1} in tap water.

Elemental distribution patterns in leaves of a plant exposed to 10 mg.L^{-1} during 72 hours is displayed in Fig. 6. Ca and Cl distribution indicate the structure of a leaf cross-section. If mercury is found throughout the leaf, its distribution is heterogeneous. For example, the concentration of Hg was higher near the lower surface (4.10 mg.g^{-1}) than in chlorenchyma (2.23 mg.g^{-1}). This is a coherent result, considering that the lower surface is in contact with water.

An important aspect of this study was the localisation of mercury within the chlorenchyma. It is interesting to note that the highest concentrations of Mn in the chlorenchyma (Mn X-rays map) would correspond to the oxygen evolving Mn_4CaO_5 cluster (OEC) in photosystem II (Rossini & Knapp, 2017) and mercury is detected at this level.

In another hand, it is well known the toxicity of mercury on photosystem II (Deng *et al*., 2013). As a result, plants exposed to Hg^{2+} (10 mg.L^{-1}) turned brown (Fig. 7b). The control of viability shows as expected that plants remain green when exposed to the same water without mercury (Fig. 7a).

The brown colour suggests that the replacement of magnesium from chlorophyll by mercury is not taking place since, heavy metal chlorophylls are reported to be more stable and, even dead plants remain green (Kupper *et al.*, 1996).

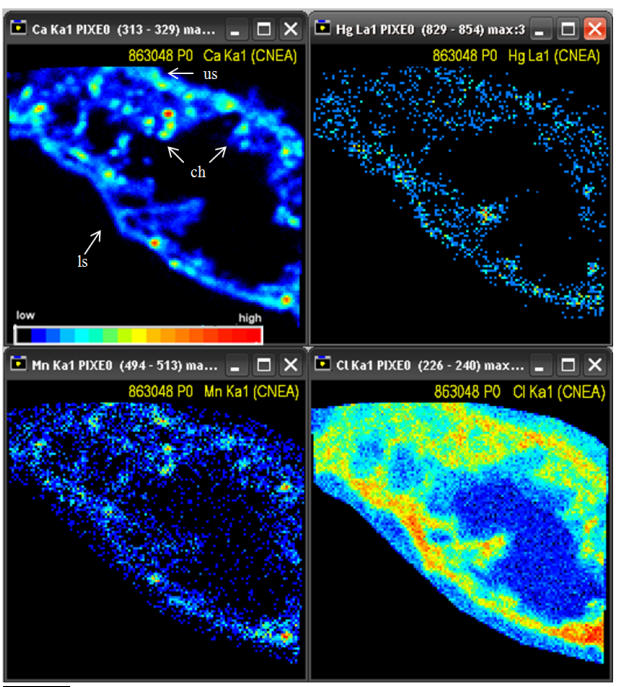

Fig. 6. 2D X-rays maps corresponding to Ca, Hg, Mn and Cl distribution from the top cross-section of a leaf of *S. intermedia* exposed to Hg^{2+} (10 mg.L^{-1}) during 72 hours. MicroPIXE conditions: scan size 300×300 μm², spot size ~ 3×3 μm². Same color scale as Fig. 3. ls: lower surface; ch: chlorenchyma; us: upper surface. Scale= 50 μm.

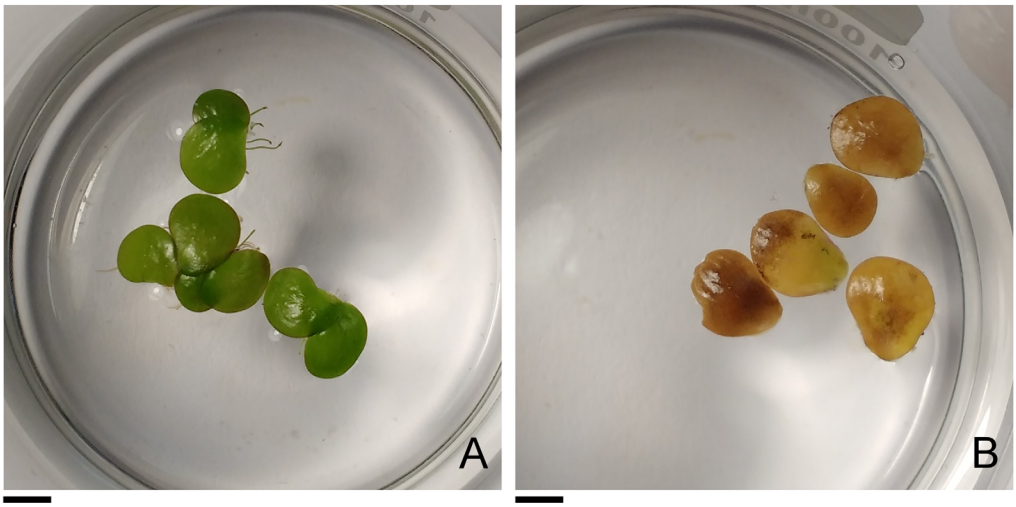

Fig. 7. *Spirodela intermedia* after a week of exposure. **A**: Hg^{2+}-free control of viability. **B**: Plants treated with aqueous Hg^{2+}(10 $mg.L^{-1}$). Scales= A-B: 4 mm.

Conclusions

Phytoremediation of aqueous Hg^{2+} by the floating autochthonous macrophyte *Spirodela intermedia* is a convenient alternative. For an initial concentration of Hg^{2+} ranging 1−10 $mg.L^{-1}$, plants behave as hyperaccumulators and reach the maximal bioconcentration in only 96 h. Exposed to an initial concentration 10 $mg.L^{-1}$, plants must be collected at the fourth day to avoid Hg leakage due to intoxication.

Since the experiments were performed using mineral water, the use of *S. intermedia* becomes an advisable procedure of removing aqueous Hg^{2+} at pilot and field scale.

Low mass solid wastes are produced on account of a high Hg/biomass proportion.

It was found that Hg^{2+} distribution in leaves is more heterogeneous than in roots. The simultaneous mapping of various elements can be used to explore the physiological mechanisms that allow these plants to accumulate and in some cases hyperaccumulate Hg^{2+}.

The temporal evolution of Ca^{2+} uptake as a mechanism to reduce the toxic effect of the incorporation of Hg^{2+} should be studied for concentrations below than 10 $mg.L^{-1}$ in order to analyze the behaviour of this uptake for longer survival times.

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Author contributions

EMF conceived, designed and carried out all the experiments, performed data analysis and wrote the paper. NAV contributed to microPIXE irradiations especially monitoring acquisition process. NAM contributed substantially to the microbeam line maintenance, software update installation and image processing. RAP supervised biological protocol design. MED conceived and designed microPIXE protocols from sample preparation to irradiation conditions, performed data analysis and wrote as well this work. MED and RAP were director and co-director, respectively, of EMF.

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