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Phytofiltration of As^{3+}, As^{5+}, and Hg by the aquatic macrophyte *Potamogeton pusillus* L, and its potential use in the treatment of wastewater

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ABSTRACT

The aim of this paper was to investigate the capacity of the aquatic macrophyte *Potamogeton pusillus* to remove As^{3+}, As^{5+}, and Hg from aqueous solutions. The plants were exposed to 0 $mg.L^{-1}$, 0.1 $mg.L^{-1}$, 0.5 $mg.L^{-1}$, 1 $mg.L^{-1}$, or 2 $mg.L^{-1}$ of As^{3+}, As^{5+}, and Hg for 20 days. The results obtained for the individual removal of As^{3+}, As^{5+}, and Hg from water solutions, together with their accumulation in *P. pusillus*, indicate that this plant can be effectively used for the removal of Hg and of moderate concentrations of As^{3+} or As^{5+} (0.1 $mg.L^{-1}$) from aquatic systems. Roots and leaves accumulated the highest amount of As when the plant was exposed to As^{5+}, but when it was exposed to As^{3+}, the root accumulated the highest amount of As, and the leaves, the highest amount of Hg. When compared to other aquatic plants species, the results showed that *P. pusillus* demonstrated a higher Hg accumulation (2465 ± 293 $\mu g.g^{-1}$) when the transfer coefficient was 40,580 ± 3762 $L.kg^{-1}$, showing the great potential of this macrophyte for phytoremediation of water contaminated with Hg. To the extent of our knowledge, this is the first report on bioaccumulation of As^{3+}, As^{5+}, and Hg by *P. pusillus*.

KEYWORDS

aquatic plant; bioaccumulation; metal; metalloid; photosynthesis

Introduction

Inorganic chemicals, such as metals and metalloids, can be found in untreated wastewater, which usually comes from residential and industrial sources. Various environmental and economic problems can be caused by these elements. Most environmental pollutants have destructive effects on soil and water quality, plant and animal nutrition, as well as on human health (Rezania *et al.* 2016).

Contamination of the aquatic environment by metals and As has become a serious concern in developing countries. Unlike organic pollutants, As and Hg cannot be easily degraded, thus enabling their persistence in nature. Their removal from contaminated water has been of utmost importance to minimize their impact on ecosystems. Although different physical, chemical, and biological approaches have been employed for this purpose, phytoremediation is one way to solve this kind of pollution problem using plants. In this process, pollutants are collected by plant roots and accumulated in the plant tissues. Thus, phytoremediation is environment-friendly, inexpensive, and it can be carried out in polluted places (remediation *in situ*) (Ali *et al.* 2013).

Several aquatic macrophytes have been used for the removal of As (Islam *et al.* 2015; Zhang *et al.* 2011; Alvarado *et al.* 208; Mishra *et al.* 2008) and Hg (Moreno *et al.* 2008; Bennicelli *et al.* 2004; Skinner *et al.* 2007; Teles Gómes *et al.* 2014) from wastewater. Aquatic macrophytes have great potential for accumulating metals and metalloids inside the plant body. In recent years much attention has been given to wastewater treatment, with the help of aquaculture (growth of aquatic plants having economic values) and the recycling of treated water for different uses. After treatment, these aquatic plants can be used for biogas production, compost production, solid waste amendments, and as fiber (Mishra *et al.* 2008).

We decided to study the bioaccumulation of As and Hg by *Potamogeton pusillus*, considering that it is a native macrophyte, with ecological importance within sub-tropical aquatic ecosystems, providing shelter and habitat for young fish and other aquatic animals in addition to its wide presence in wild and urban habits of Argentina (Monferran *et al.* 2009). Although some studies have been carried out to determine the capability of *Potamogeton pusillus* to accumulate Cu^{2+} and Cr^{6+} (Monferran *et al.* 2012), no information is available on the capability of this macrophyte to bioaccumulate As or Hg.

The main aim of this study was to evaluate the bioaccumulation processes and tolerance of *P. pusillus* upon individual exposure to As^{3+}, As^{5+}, and inorganic Hg (as $HgCl_2$). We also analyzed some cellular changes induced upon exposure to As^{3+}, As^{5+}, and Hg including the measurement of protein and chlorophyll-a contents, which have been shown to be a reliable method to assess physiological stress in aquatic macrophytes (Monferran *et al.* 2009; 2012).

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Supplemental data for this article can be accessed on the publisher's website.

Materials and methods

Reagents and materials

All reagents were of analytical grade supplied by Sigma–Aldrich and Sintorgan (Argentina). All materials used were left with sulfuric-nitric acids solution overnight and then washed with ultra-pure water to avoid contamination. Agilent Technology Multi Element Calibration standard solution (10 mg.L^{-1} in 1% nitric acid) was used as stock solution for calibration of metal quantification equipment.

Equipments

Reverse osmosis (RO) water was obtained from an Arium 61316-RO equipment (Sartorius, Germany). Ultra-pure water (TOC < 5 μg.L^{-1}) was obtained from an Arium 611 UV purification system (Sartorius, Germany).

Absorption of chlorophylls and proteins was recorded using an UV-Vis spectrophotometer (Shimadzu Corporation MultiSpec-1501), equipped with multiple-thermostatized cell holder.

Quality assurance and quality control

All samples were digested in triplicate, ($n = 5$, three plant per beaker, 5–8 g fw per liter, this means that each n was digested three times). When samples were analyzed by ICP-MS, the mean of three runs was obtained for each sample. Concentrations of elements were determined in triplicate. Quality assurance (QA) and quality control (QC) of elements analysis were done using certified reference material (CRM): NIST 1547 (Peach Leaves). Recoveries from CRM were 93 ± 15% for Hg. Spiked samples were also prepared. Variable amounts of mixed standard solutions, containing all elements analyzed, were added to 0.02–0.04 g of plant sample prior to sample digestion to double the starting concentration for each element. The rest of the procedure was the same as used for non-spiked samples. The average recovery was 95 ± 6% and 103 ± 10% for As and Hg, respectively.

Plant material and experimental setup

Macrophytes *P. pusillus* were collected from a reference site (Córdoba, Argentina; Monferran *et al.* 2012). Plants were acclimated during two weeks in 40 L glass aquaria filled with 10% Hoagland's solution and sediment (1/4) from the same sampling area. During two weeks, they were maintained at constant laboratory temperature (25°C), under a light/dark regime, 14 h:10 h photoperiod (Monferran *et al.* 2012).

During exposures, plants of *P. pusillus* were kept in 1.5 L beakers ($n = 5$, three plant per beaker, 5—8 g fw per liter) and maintained in 10% Hoagland's solution.

Experiments were carried out using either As^{3+}, As^{5+}, or Hg^{2+}, prepared from $NaAsO_2$ (Sigma–Aldrich), $Na_2HAsO_4.7H_2O$ (Biopack) and $HgCl_2$ (Merck), respectively. The experimental design involved six experimental conditions for each element: control 1: a plant control, consisting of plants grown in 10% Hoagland medium without the addition of metals; control 2: a metal control, consisting of metal solutions without plants; and plants exposed to 0.1, 0.5, 1, and 2 mg.L^{-1} of As^{3+}, As^{5+}, or Hg^{2+}individually. The experiments mentioned above were carried out during 7, 14, and 20 days of exposure. This first experiment was done with the purpose of evaluating the bioaccumulation capacity of the plant, for this reason, changes in soluble metal concentration from experimental tanks were measured before starting each experiment (t_0) and every two or three days, until final experiments. All exposures were done in five replicates ($n = 5$, which means 5 plants tested at each concentration). At the end of exposure, macrophytes were washed thoroughly with ultra-pure water. Washed plants were then used to measure the bioaccumulation (considering the whole plant).

Afterwards, the differential accumulation pattern between different parts of the aquatic plant (roots, leaves and stems) was measured. Thus, *P. pusillus* was exposed to 0.1, 0.5, 1, and 2 mg.L^{-1} for individual metal exposures during 15 days. At the end of exposure, macrophytes were washed thoroughly with ultra-pure water, weighed and dissected (leaf, stem, and root).

Finally, in order to evaluate the toxicity of As^{3+}, As^{5+}, or $Hg^{2+}$to *P. pusillus*, the macrophytes were exposed to: 0.1, 0.5, 1, and 2 mg.L^{-1} for individual metal exposures, during 5, 10, and 15 days. At the end of exposure, macrophytes were washed thoroughly with ultra-pure water. Finally, samples were frozen with liquid nitrogen and kept at −80 °C until protein and chlorophyll analysis.

Arsenic and mercury analysis in plants

During exposures to single metal solutions, the amount of metal accumulated by the whole plant was evaluated at days 7, 14, and 20. For that purpose, plants were dried in an oven at 40 °C to a constant weight before being ground and homogenized with a mortar and pestle. Plant samples were mineralized using Teflon tubes. For Hg analysis, approximately 25 mg of sample was weighed and digested with 6 mL of HNO_3 (sub boiling grade) and 2 mL of HCl (ultrapure), in closed Teflon tubes on heating plates set at 220 °C, during 8 h. For As analysis, 25 mg of sample was weighed and digested with 8 mL of HNO_3 (sub boiling grade) also in closed Teflon tubes on heating plates set at 220 °C, during 8 h. Mineralized samples were quantitatively transferred to 10 mL volumetric flasks, completing the volume with HNO_3 2%, followed by filtration using 0.45 μm filters. All samples were stored at 4°C until analysis. The multi-elemental analysis in both abiotic and biotic samples was performed using a Mass Spectrometer Inductively Coupled Plasma (ICP-MS), (Q-ICPMS, Agilent Technology 7500 cx Series, California), equipped with an ASX-100 autosampler (CETAC Technologies, Omaha, NE, USA).

Analysis of plants for biochemical parameter

Chlorophyll (Chl) concentrations were determined in leaves of *P. pusillus* according to Monferran *et al.* (2012). Concentrations of pigments in plant extracts were measured by visible spectrophotometry using a microplate reader (Chl = 649 and 665 nm). Concentrations were calculated and reported in mg chlorophyll g^{-1} ww.

The amount of protein was determined in whole plant material of *P. pusillus*, according to Monferran *et al.* (2012). Briefly,

0.5 g plant were shock-freezed in liquid nitrogen, homogenized using aceramic mortar, and dispersed in 5 mL 0.1 M potassium phosphate buffer, pH 7.5. This solution was centrifuged at 26,000 g for 20 min. The remaining supernatant was used for protein measurement by Bradford's method (1976).

Statistics

Data were expressed as the average ± standard deviation (SD) in tables and graphics. The data satisfy the assumptions (normality and variance homogeneity) of an analysis of variation (ANOVA), so a posteriori test, LSD Fisher test, with the corresponding Bonferroni errors correction, was used to look for significant differences between the means of control and other treatments ($p < 0.01$). InfoStat Software (V1.1) was used for all statistical calculations.

Results and discussion

Individual removal of As^{3+}, As^{5+}, and Hg from water solutions

Experiments containing *P. pusillus* and As^{3+}or As^{5+} in solutions do not evidence significant changes in As concentration in any of the speciations of the metalloid evaluated. The concentrations of As^{3+} or As^{5+} in metalloid control (experiment without plant) were almost constant during the time that the experiment lasted (supporting information). In Table 1, we can see that the removal percentage of As^{3+} and $As^{5+}$was the highest (13.8% and 20.7%, respectively) at the lowest concentration tested (0.1 $mg.L^{-1}$), but at higher exposure concentrations, the removal percentage decreases to values lower than 10% for both As species.

Figure 1A and B shows that the accumulation of $As^{3+}$and As^{5+} by *P. pusillus* increased as the exposure concentration increased, but it did not increase as the exposure time increased, this means that the concentration of As accumulated by *P. pusillus* when it was exposed for 7 days at different As^{3+}or As^{5+} concentrations was the same or did not have statistically significant differences than when it was exposed for 14 or 20 days to the same concentrations mentioned above.

P. pusillus was able to accumulate more As concentrations when it was exposed to a same concentration of As^{3+}than to As^{5+}. For example: *P. pusillus* was able to accumulate 281 $\mu g.g^{-1}$ of As when it was exposed to 2 $mg.L^{-1}As^{3+}$, but it accumulated 117 $\mu g.g^{-1}$ of As when it was exposed to the same concentration of As^{5+}. Similar concentrations differences were observed when *P. pusillus* was exposed to 0.1; 0.5 or 1 $mg.L^{-1}$ of As^{3+}or As^{5+}. These results are important given that As^{5+} is more toxic than As^{3+}. In a thorough review about As phytoremediation using macrophytes (Rahman and Hasegawa 2011), results for As uptake from different aquatic plants were compiled. Several authors have reported that some species of aquatic macrophytes accumulate high amounts of As from water (Robinson *et al.* 2005; Alvarado *et al.* 2008; Mishra *et al.* 2008; Zhang *et al.* 2011). Working with an initial concentration of 0.15 $mg.L^{-1}$ and 21 days of exposure (arsenic speciation was not specified), Alvarado and coworkers have found a removal efficiency of 18% for *Eichhornia crassipes* and 5% for *Lemna minor*, (values close to our removal efficiency for *P. pusillus*) (Alvarado *et al.* 2008). Other authors have reported much higher values. In fact, Mishra and coauthors studied and compared the removal efficiency for As (arsenic speciation was not specified) in *E. crassipes, L. minor*, and *Spirodela polyrrhiza*, finding values of 80%, 60%, and 40%, respectively. In that work, an initial As concentration of 0.05 $mg.L^{-1}$ was used. High removal efficiency (90%–100%) has also been reported for *Micranthemum umbrosum* when it was exposed to 0.2 and 0.5 $mg.L^{-1}As^{3+}$ during 7 days (Islam *et al.* 2015). We did not find any work comparing the accumulation capacity of $As^{3+}$and $As^{5+}$by the same aquatic plant species.

Experiments containing *P. pusillus* and Hg in solutions show a drop in metal concentration from the beginning of the experiment until day 20 for all the concentrations evaluated. Metal control (without plants) was performed under the same conditions as plant exposures. The purpose of this test was to evaluate a possible loss by volatilization of the studied compounds. Decrease in Hg concentration in solution does not occur in the absence of *P. pusillus*, and there is a slight loss by volatilization (~15%), although the disappearance rate of Hg in solution in the presence of the macrophyte exceeds the rate of volatilization under the conditions tested (supporting information). Furthermore, the removal of Hg by *P. pusillus* was higher than that reported for As species (Table 1), the removal percentage was almost constant throughout the evaluated concentrations (97.7%–99.8%), observing a minimum decrease in the removal efficiencies for Hg (2%), as the exposure concentration increased.

The accumulation of Hg by *P. pusillus* after 7, 14, and 20 days of hydroponic treatment with different concentrations of Hg is shown in Figure 1C. A trend to increase metal accumulation is observed as metal concentrations in solution increased. The maximum rate of metal accumulation was found after day 7 at 2 $mg.L^{-1}$ Hg, when 96% of the total accumulated metal was taken up by the plant (2372 $\mu g.g^{-1}$ dw). Metal accumulation continues at days 14 and 20, although the bioaccumulation rate was lower than that reported for day 7 (Figure 1C). Thus, Hg content was 2034 $\mu g.g^{-1}$ dw (83%) after a 14-day exposure, and 2465 $\mu g.g^{-1}$ dw after a 20-day exposure. It is worth to mention that bioaccumulation also occurred at lower Hg concentrations (0,1; 0,5 and 1 $mg.L^{-1}$) but at significantly lower rates in comparison with the bioaccumulation observed during exposures at 2 $mg.L^{-1}$ (Figure 1C).

Table 1. Removal of As^{3+}, As^{5+}, and Hg from water solutions by *P. pusillus* after a 15-day exposure. ($n = 5$).

As^{3+} ($mg.L^{-1}$)	As^{5+} ($mg.L^{-1}$)	Hg ($mg.L^{-1}$)	% Removal
0.1			13.8
0.5			1.6
1			7.4
2			5.0
	0.1		20.7
	0.5		3.9
	1		0.0
	2		1.2
		0.1	99.8
		0.5	98.8
		1	98.6
		2	97.7

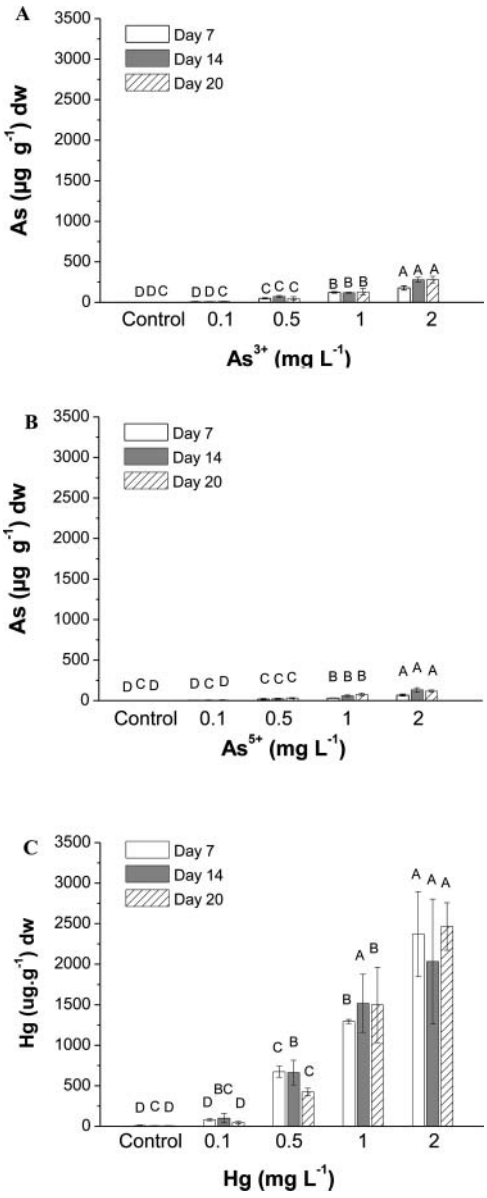

Figure 1. Time and concentration-dependent accumulation of As^{3+} (A), As^{5+} (B), and Hg (C) in *P. pusillus* ($n = 5$). Values are reported as mean ± SD. Different letters indicate significantly different values at a particular time with respect to control group (DGC, $p \leq 0.01$).

Accumulation of Hg by macrophytes was also reported by Samecka-Cymerman and Kempers (1996) who used *Scapania undulata* to remove Hg from wastewaters (0.005 mg.L^{-1}) of pesticide factories during 14 days of observation. Initial and final Hg concentrations in plant tissue samples were 0.05 μg.g^{-1} and 2.40 μg.g^{-1}, respectively. In a hydroponic system, Qian *et al.* (1999) cultivated 12 plant species for the treatment of mercury-contaminated water (1 mg.L^{-1}). After 10 days of experiment, the metal concentration in the aerial part of the plants attained 5 μg.g^{-1} (for *Pistia stratiotes* L and *Wedelia trilobata* Hitchc), and 90 μg.g^{-1} (for *Sesbania drummondii*). Moreno *et al.* (2008) evaluated the phytofiltration of mercury-contaminated water by *Brassica juncea* (L.). The final Hg concentrations in root plus shoot plant tissue samples after 5 days of exposure to 0,5; 1 and 2.5 mg.L^{-1} Hg solutions were 137, 143, and 330 μg.g^{-1}, respectively. Skinner *et al.* (2007) evaluated the effectiveness of four aquatic plants including water hyacinth (*E.crassipes*), water lettuce (*Pistia stratiotes*), zebra rush (*Scirpus tabernaemontani*), and taro (*Colocasia esculenta*) for their capabilities for removing 2 mg.L−1 Hg from water. The Hg concentration in the plants after 30 days of exposure was 26; 83; 3,9 and 7 mg.g^{-1}, respectively. Regier *et al.* (2013) demonstrated the accumulation of Hg in shoots of *Potamogeton pectinatus* and *Potamogeton nodosus* collected at different sampling sites in the Babeni reservoir (Romania), 2.24 and 0.19 μg.g^{-1}, respectively. We did not find any work reporting the accumulation capacity of As^{3+}, As^{5+}, and Hg by *P. pusillus*.

The present work showed a higher Hg removal efficiency than all related studies previously reported. Moreover, Hg uptake by this plant is also higher than other metal uptakes by the aerial part of the plant and it also has higher transfer coefficient (TC), as previously reported (Moreno *et al.* 2008). The water–plant TC was calculated according to Moreno *et al.* (2008), who defined it as the relation between the Hg concentration in the plant (μg. Kg^{-1} dw) and the Hg residual concentration in exposure water (μg. L^{-1}) measured at the different exposure days (5, 10, 15, and 20 days) as a proper way to express the relative metal absorption by *P. pusillus*. The Hg concentration background in plant samples was 8.9 ± 5.2 μg.g^{-1}, attaining 2465 ± 293 μg.g^{-1} after an exposure time of 20 days (Figure 2), corresponding to the maximum transfer coefficient of 40,580 ± 569.5468 L.kg^{-1}.

The work results showed that the *P. pusillus* was capable of accumulating more Hg than the species used by Samecka-Cymerman and Kempers (1996), Qian *et al.* (1999), Moreno *et al.* (2008)Skinner *et al.* (2007), Benicelli *et al.* (2004) and Teles Gómes *et al.* (2014).

Bioconcentration factor

Phytofiltration capacity can be quantified by calculating the bioconcentration factor (BCF), this factor indicates the capability of a plant species for accumulating a metal into its tissues from the surrounding environment. It is calculated as follows:

$$BCF = C_{ss}/C_w$$

where C_{ss} is the element concentration in the plant harvested tissue (μg.g^{-1}, dry weight), and C_w is the element concentration in water (mg.L^{-1}).

BCF is a more important measure than shoot metal concentration when considering the potential of a given candidate species for phytofiltration (Sakakibara *et al.* 2011). According to Yoon *et al.* (2006), only plant species with BCF greater than 1 have the potential to be used for phytofiltration. Generally, BCF values of As^{3+} were higher than 100 for all concentrations evaluated, but values of As^{5+} were between 58% and 77%. The results of BCF for the exposures to As^{3+} or As^{5+} obtained in this work are lower than those obtained by Favas *et al.* (2012) who reported a BCF of 3300 for *P. pusillus* L in field conditions with an average As concentration in water of 25.9 μg.L^{-1}. For Hg, the BCF of all concentrations evaluated was much higher (in the order of 1000) than that of the As species exposure

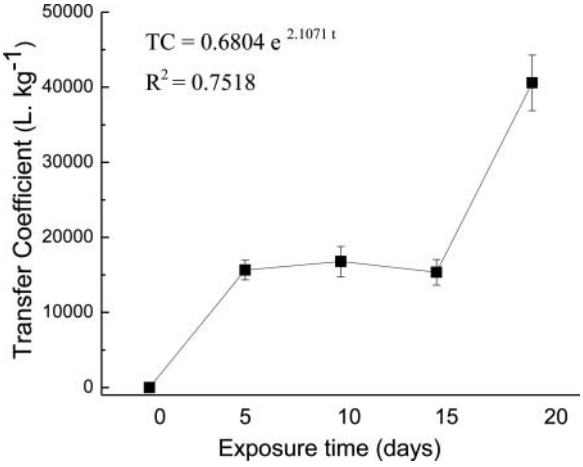

Figure 2. Variation of the water–plant transfer coefficient of Hg (exposure to 2 mg. L^{-1}) as a function of exposure time ($n = 5$).

(supporting information). These results are in agreement with the accumulation pattern of the elements evaluated in this study.

Distribution of As $^{3+}$, As $^{5+}$, and Hg in plant tissues

Table 2 shows bioaccumulation patterns for As^{3+}, As^{5+}, and Hg in leaves, stems, and roots of *P. pusillus*. Our current results show that both As and Hg are distributed throughout the plant, although we cannot ensure the oxidation state of As within the plant. Present results corroborate earlier findings showing that Cu and Cr are directly taken up by leaves, stems, and roots of *P. pusillus* (Monferran *et al.* 2012). Relative amounts of As^{3+}, $As^{5+}$and Hg increased in all studied tissues as metal concentration increased, showing significantly higher levels of As in root than in shoots or leaves when the aquatic plant was exposed to all concentrations of As^{3+}evaluated. On the other hand, when *P. pusillus* was exposed to As^{5+}, the plant tissue with the highest As accumulation was the root at the lowest concentration exposed (0.1 mg.L^{-1}), the stems at 0.5 mg.L^{-1}, and no statistically significant differences were observed between the plant parts when it was exposed to 1 and 2 mg.L^{-1} (Table 2). Finally, when *P. pusillus* was exposed to Hg concentrations, we observed that the highest levels of Hg were found in leaves than in shoots or roots at all concentrations evaluated. These results indicate that the ability of *P. pusillus* to concentrate studied metals from the external medium varies in the different plant parts, and it also fluctuates between both studied metals.

Table 2. Mean concentration of As^{3+}, As^{5+}, and Hg in leaves, roots, and stems of *P. pusillus* after 15-day exposures ($n = 5$). Values are reported as mean ± SD. Different letters indicate significantly different values at a particular concentration between different plant parts (leaves, stems, and roots) (DGC, $p \leq 0.01$).

		Metal concentration in plants (μg. g^{-1} dry weight)		
Initial concentration (mg. L^{-1})	Element	Roots	Stems	Leaves
Control	As $^{3+}$	<LOD	<LOD	<LOD
0.1	As $^{3+}$	3.84 ± 1.67 ^{b}	5 ± 1 ^{b}	27.6 ± 0.2 ^{a}
0.5	As $^{3+}$	48 ± 5 ^{b}	20 ± 1 ^{c}	82 ± 5 ^{a}
1	As $^{3+}$	78 ± 4 ^{b}	52 ± 3 ^{b}	239 ± 58 ^{a}
2	As $^{3+}$	109 ± 2 ^{b}	104 ± 2 ^{b}	216 ± 2 ^{a}
Control	As $^{5+}$	<LOD	<LOD	<LOD
0.1	As $^{5+}$	5 ± 1 ^{b}	1.1 ± 0.2 ^{c}	14 ± 1 ^{a}
0.5	As $^{5+}$	72 ± 7 ^{a}	18.1 ± 0.4 ^{b}	29 ± 5 ^{b}
1	As $^{5+}$	90 ± 9 ^{a}	46 ± 20 ^{a}	51 ± 3 ^{a}
2	As $^{5+}$	218 ± 34 ^{a}	196 ± 23 ^{a}	189 ± 85 ^{a}
Control	Hg	1.09 ± 0.9 ^{a}	0.5 ± 0.2 ^{a}	1.3 ± 0.4 ^{a}
0.1	Hg	15 ± 3 ^{b}	61 ± 17 ^{a}	44 ± 6 ^{a}
0.5	Hg	78 ± 17 ^{c}	378 ± 56 ^{a}	256 ± 58 ^{b}
1	Hg	121 ± 10 ^{c}	740 ± 140 ^{a}	455 ± 138 ^{b}
2	Hg	401 ± 140 ^{b}	1246 ± 304 ^{a}	659 ± 240 ^{b}

Previous studies have shown that roots are the main tissue for the accumulation of various metals by aquatic plants (Lesage *et al.* 2007; Peng *et al.* 2008), in good agreement with our current results where *P. pusillus* was exposed to $As^{3+}$and to the lowest concentration of As^{5+}. Three mechanisms have been proposed for the uptake of As species in aquatic macrophytes – (i) active uptake through phosphate uptake transporters, (ii) passive uptake through aquaglyceroporins, and (iii) physicochemical adsorption on root surfaces. Plants mainly uptake As^{5+} through phosphate uptake transporters (Azizur Rahman 2011); however, physicochemical adsorption on root surfaces has also been supposed to be an alternative uptake pathway for this As species (Robinson et al. 2005; Rahman et al. 2011). As^{3+} gets into the plants by a passive mechanism through the aquaglyceroporin channels (Zhang *et al.* 2011). A lower accumulation of As species in leaves than in root can be due to the plant's role in protecting photosynthesis from toxic levels of trace elements (Mishra *et al.* 2008).

Metal accumulation in leaves may be largely attributed to ion exchange within this tissue and the surrounding solution, and also via passive penetration of ions into the peripheral region. Uptake by leaves becomes important when the metal concentration in the surroundings is high (Demirezen and Aksoy 2004).

On the other hand, *P. pusillus* was able to accumulate higher concentrations of Hg in leaves than in shoot or roots, contrary to the results by Malar *et al.* (2015) who reported that the root was the part of *E. crassipes* seedlings where most Hg was accumulated when exposed to 50 mg. L^{-1}. Furthermore, cell walls of leaves are rich in organic compounds like pectins, formed by polymers of negatively charged polygalacturonic acid. These negatively charged compounds have great affinity for cations as Hg^{2+}, and therefore the adsorption of the metal with subsequent absorption can occur (Rengel 1989).

Stems were the part of *P. pusillus* with less As and Hg accumulation, a possible explanation for this could be that stems represent about 10% of the whole plant material, thus having a smaller contact surface with the medium than roots and leaves. Our results are in agreement with those published by Monferran *et al.* (2012), who observed that roots and leaves accumulated the highest amount of Cu and Cr in *P. pusillus* followed by stems.

Considering the high and moderate capacity of *P. pusillus* to phytoextract Hg and As, respectively, and its easy harvest and wild distribution in South America, this plant species can be considered an ideal candidate to be used in phytoremediation of contaminated water bodies and wastewater effluents containing Hg and As.

3.4. Accumulation of metals in P. pusillus, impact on biochemical parameters

The bioaccumulation of metals in higher plants is often accompanied by the induction of a variety of cellular changes, some of which directly contribute to the metal tolerance capacity of plants. Among the variety of targets reported for metals in plants, the photosynthetic apparatus seems to be the most sensitive.

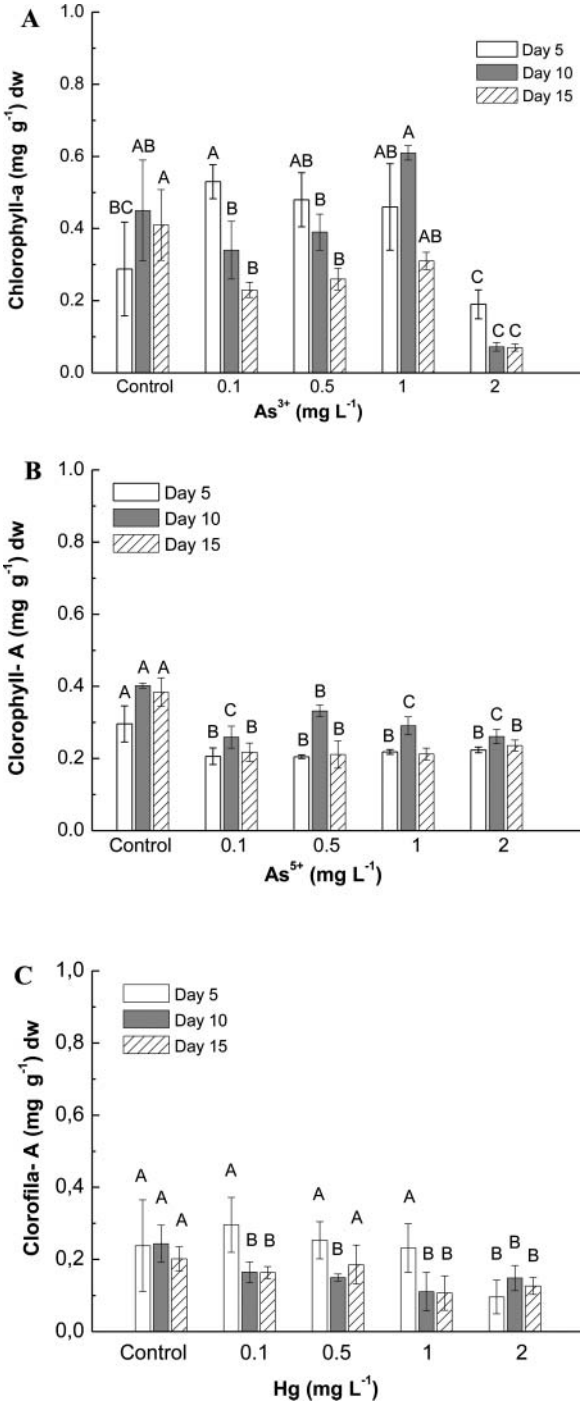

Figure 3. Contents of Chlorophyll-a in *P. pusillus* exposed to different concentrations of As^{3+} (A), As^{5+} (B), and Hg (C) at different times ($n = 5$). Values are reported as mean ± SD. Different letters indicate significantly different values at a particular time with respect to control group (DGC, $p \leq 0.01$).

NOEC and LOEC values were calculated considering chlorophyll-a and protein contents in *P. pusillus* exposed to different concentrations of As^{3+}, As^{5+}, and Hg during 15 days. The toxic effect of As and Hg on chlorophyll-a and protein contents varied depending on the metal used for exposures. Chlorophyll-a decreased as the As^{3+}, As^{5+}, and Hg concentration increased (Figure 3A–C). NOEC for chlorophyll-a was 0.1 mg. L^{-1} for As^{3+}, As^{5+}, and Hg. This indicates that As species are more toxic to the plant than Hg, taking into account that the amount of Hg accumulated (46 $\mu g.g^{-1}$) at 0.1 $mg.L^{-1}$ of exposure is much higher than that of As (10 and 6 $\mu g.g^{-1}$ for As^{3+} and As^{5+}exposure, respectively). It was not possible to calculate NOEC at the concentrations evaluated in this study when protein contents were used because there were no statistically significant differences between the exposed concentrations in this study of As^{3+}, As^{5+}, and Hg and the control during the 15 days of exposure (supporting information).

Comparing these results, it seems that As^{3+}, As^{5+}, and Hg have a more toxic effect on chlorophyll-a than on proteins level (NOEC = 0.1 $mg.L^{-1}$). In our experiments, significant damages in macrophyte pigments have been observed in *P. pusillus* after exposure to As^{3+}, As^{5+}, and Hg (Figure 3A–C). These changes reflect the intensity and diversity of the disorders generated by these elements ions in cell metabolism. Loss of photosynthetic pigments is a common response of plants to stress (heat, diseases, and pollution), which has been observed after As treatment in lichen *Xanthoria parietina* (L.) (Pisani *et al.* 2011), in the aquatic plants *Pistacia lentiscus*, and *Tamarix gallica* (Moreno-Jiménez *et al.* 2009), and *Cucumis sativus* (Cargnelutti *et al.* 2006). In plants, Hg ions may substitute metal ions in photosynthetic pigments, causing a decrease in photosynthesis rates (Xylander *et al.* 1996). Several studies have shown that Hg in the substrate decreased the levels of photosynthetic pigment chlorophylls at a prolonged exposure. It also strongly inhibits the photosynthetic electron transport chain, where photosystem II (PS II) is the most sensitive target (Bernier and Carpentier 1995). Arsenic has many properties of heavy metals, so its toxic effects are similar to those of Pb and Hg. The availability of As uptake varies depending on the arsenic species. Dimethyl arsonic acid has the lowest availability, followed by monomethyl arsonic acid as As^{5+}, and with As^{3+} having the highest bioavailability (Islam *et al.* 2015). Reduction in chlorophyll content may be attributed to an impaired uptake of inorganic elements, damage of photosynthetic apparatus, or chlorophyll degradation by increased chlorophyllase activity (Mishra *et al.* 2008).

There were no significant differences in protein levels when *P. pusillus* was exposed to individual As^{3+}, As^{5+}, or Hg solutions for 15 days (see supplementary material Figure 4A–C).

Conclusions

To our knowledge, this is the first report on bioaccumulation of As^{3+}, As^{5+}, and Hg by *P. pusillus*. The results obtained for the individual removal of As^{3+}, As^{5+}, and Hg from water solutions together with their accumulation in *P. pusillus* indicate that this plant could be used for the removal of Hg, and of low concentrations of As^{3+} or As^{5+} (0.1 $mg.L^{-1}$) from wastewaters. Upon

15 days of exposure to 0.1 mg. $L^{-1}As^{3+}$, As^{5+}, or Hg, chlorophyll-a levels were reduced, evidencing metal toxicity to the plant. However, exposure to 0.1 mg.$L^{-1}As^{3+}$, As^{5+}, or Hg did not evidence such toxicity in protein contents.

Regardless of the metal concentration used, roots presented a higher As accumulation when the plant was exposed to As^{3+}, root and leaves when it was exposed to As^{5+}, and only leaves when it was exposed to Hg, suggesting that the part of the plant where the element will accumulate depends on the element to which the plant is exposed.

These results are interesting if we consider that previous studies reported less Hg accumulation capacity for different plant species than those reported in this work by *P. pusillus*. Our study contributes with new data on the use of aquatic plants for phytoremediation of Hg and As. Further work is in progress to understand the molecular and biological mechanisms by which *P. pusillus* is able to accumulate large amounts of Hg in its tissues.

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Supporting information is shown in Figure S1: Time-dependent removal of As^{3+}, As^{5+} and Hg from aqueous solutions by *P. pusillus*; Figure S2: Contents of total proteins in *P. pusillus* exposed to different concentrations of As^{3+}, $As^{5+}$and Hg; and Table S1:Bioconcentration factor (BCF) for As^{3+}, As^{5+} and Hg in *P. pusillus*.

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