

Single and combined phytoextraction of lead and cadmium on submerged plants *Potamogeton pusillus* L.: removal, bioaccumulation pattern, and phytotoxicity

Cynthia B Rivela

Universidad Nacional de Cordoba Facultad de Ciencias Quimicas

Julieta Griboff

Universidad Nacional de Cordoba Facultad de Ciencias Quimicas

Daniela S. Arán

Universidad Nacional de Cordoba Facultad de Ciencias Exactas Fisicas y Naturales

Facundo L Cortés

Universidad Nacional de Cordoba Facultad de Ciencias Quimicas

M. Eugenia Valdes

Universidad Nacional de Cordoba Facultad de Ciencias Quimicas

Carlos A Harguinteguy

Universidad Nacional de Cordoba Facultad de Ciencias Exactas Fisicas y Naturales

Magdalena V. Monferran

`mmonferran@fcq.unc.edu.ar`

Universidad Nacional de Cordoba Facultad de Ciencias Quimicas

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Abstract

Under the present investigation, the submerged plant *Potamogeton pusillus* has been tested for the removal of lead (Pb) and cadmium (Cd). *P. pusillus* removal efficiency and accumulation capacity were examined in separated Pb and Cd solutions, at 0.5, 1.0, and 2 mg L⁻¹, and in solutions where both metals were present at the same concentration (0.5, 1.0, and 2 mg L⁻¹), under laboratory conditions for 3, 7, and 10 days. Also, we examined the removal efficiency and accumulation capacity when a set of plants were exposed to 0.5 mg L⁻¹ of Pb (or Cd) and increasing concentrations (0.5, 1, and 2 mg L⁻¹) of Cd (or Pb) for 10 days. The effect of Cd and Pb was assessed by measuring changes in the chlorophylls, carotenoids, and malondialdehyde contents. Results showed that *P. pusillus* could accumulate Cd and Pb from individual solutions. Roots and leaves accumulated the highest amount of Cd and Pb followed by the stems. Some phytotoxic effects were observed, especially at individual Cd exposures, but these effects were not observed in the two-metal system. The removal and accumulation of Pb by *P. pusillus* were significantly enhanced in the presence of Cd under certain conditions, presenting a good alternative for the removal of these metals from polluted aquifers. To the extent of our knowledge, this is the first report on both enhanced phytoextraction of Pb in the presence of Cd and bioaccumulation of these heavy metals by *P. pusillus*.

Introduction

Nowadays, there is global concern about freshwater scarcity. Nearly 35% of the world's population may experience a water access crisis in the coming years due to the impacts of pollution, inadequate water management, and climate change on this life-essential resource (WHO 2022). Industrialization is among the major causes of environmental degradation and contamination of surface water bodies (e.g., ponds, rivers, streams), putting ecosystems and human health at risk (Song et al. 2023). Industrial wastewater is produced in large volumes and, in most cases, is discharged into the environment without being treated, releasing a wide variety of pollutants into the aquatic environment, including heavy metals and organic compounds (Batley and Campbell 2022).

Heavy metals are dangerous environmental pollutants because of their non-biodegradability, toxic effects on different organisms, and ability to bioaccumulate in aquatic ecosystems (Ali et al. 2019). Essential metals (e.g., iron, copper, molybdenum, zinc, manganese) have a range of optimal concentrations and can be dangerous at high concentrations. On the other hand, for metals such as cadmium (Cd) and lead (Pb), it is unknown if they have physiological functions in living beings, and their accumulation could cause toxic effects in them (Zamora-Ledezma et al. 2021).

Lead can be found in different products, including storage batteries, solders, ammunition, plumbing, and pigments. Affections of the gastrointestinal tract, kidneys, and central nervous system are associated with the presence of Pb in humans (Collin et al. 2022). On the other hand, Cd is widely used in the electroplating industry, rechargeable batteries, the dye industry, plastics, and metal alloys. Additionally, Cd is regarded as a carcinogenic agent and has deleterious effects on human kidneys, skeletons, and respiratory systems

(Genchi et al. 2020). This element is classified as a Group 1 human carcinogen, and it is also currently listed by the World Health Organization (WHO) as one of the ten chemicals of major public concern. Thus, Cd and Pb are considered priority water pollutants from a monitoring perspective by most countries and international organizations (Emiliani et al. 2020).

Divalent cations, Pb^{2+} and Cd^{2+} , are the most common forms in the environment, either in water or soil (Huang et al. 2020). Anthropogenic activities further intensify the coexistence of these heavy metals (Abadin et al. 2007). For example, the presence of Pb^{2+} and Cd^{2+} has been recorded in effluent samples from three food industries in Nigeria, with concentrations of Pb ranging from 1.6 to 3.3 mg L⁻¹ and from 0.31 to 1.8 mg L⁻¹ for Cd (Nwosu et al. 2014). Another study found Pb in concentrations of 3.5–4.5 mg L⁻¹ and Cd in concentrations of 1.1–3.7 mg L⁻¹ in industrial waters in a metropolitan area of Bangladesh (Islam et al. 2016). In addition, Bokhari et al. (2019) reported concentrations of Pb and Cd of 1.51 mg L⁻¹ and 0.74 mg L⁻¹, respectively, in untreated wastewater from an industrial area in Pakistan. In all the cases, Pb and Cd concentrations were higher than the WHO-recommended safe limits for Pb (0.01 mg L⁻¹) and Cd (0.003 mg L⁻¹) in wastewater (Kinuthia et al. 2020). Therefore, it is critical to treat industrial wastewater before releasing it into the environment to ensure that heavy metal concentrations are within these recommended limits.

These hazardous pollutants can be eliminated from industrial effluents via conventional treatment techniques such as chemical precipitation, adsorption, ion exchange, flocculation, and electrochemical removal (Chai et al. 2021). These methods have significant drawbacks, including high operational costs, high energy requirements, and adverse effects on aquatic ecosystems (Raouf and Raheim 2016). Hence, long-term, and cost-effective heavy metal removal techniques are required.

Phytoremediation involves the removal, degradation, or stabilization of contaminants present in water, soil, air, and sediments using plants. This green technology is considered one of the safest and most ecological tools for the restoration of contaminated aquatic ecosystems (Wei et al., 2021). Aquatic macrophytes have proven to be excellent candidates for phytoremediation strategies due to their ability to act as receptacles for heavy metals. In aquatic phytoremediation systems, aquatic plants can be either floating on the water's surface or submerged in the water. The floating aquatic plants absorb or accumulate contaminants through their roots, while submerged plants accumulate metals through their whole bodies (Rahman et al. 2018). Several studies have been carried out to determine the capability of some macrophytes, viz., *Lemna minor*, *Lemna gibba*, *Spirodela polyrhiza*, and *Eichornia crassipes*, to accumulate Pb and Cd individually or mixed (Fawzy et al. 2011; Doğanlar 2013; Chaudhuri et al. 2014). On the other hand, exposure to heavy metals and accumulation of these pollutants, even at low concentrations, can lead to lethal damages in plants, e.g., breakdown of the photosynthesis apparatus and increased concentration of reactive oxygen species (ROS) leading to oxidative stress (Jonak et al., 2004).

Potamogeton pusillus L. is a native macrophyte with totally submerged or floating leaves (heterophylly) with ecological importance in sub-tropical aquatic ecosystems, present in wild and urban habitats in Argentina (Monferrán et al. 2009). The use of this aquatic plant has been investigated for the monitoring

and remediation of water bodies polluted with heavy metals like Cu^{2+} , Cr^{+6} , As^{+3} , As^{+5} , and Hg (Monferrán et al. 2009, 2012; Harguinteguy et al. 2016; Griboff et al. 2018; Bertrand et al. 2019). Local macrophyte species with an elevated biomass production rate, such as *P. pusillus*, enable more sustainable and economically viable phytoremediation strategies, especially in areas where communities have limited financial resources. To the best of our knowledge, there is no research regarding the removal efficiency, accumulation, and tolerance of Pb and Cd, either individually or combined, by *P. pusillus*. Furthermore, there is limited information on the time-dependent effects of Cd and Pb on different physiological parameters of this macrophyte.

The present research evaluates the potential use of *P. pusillus* in wastewater treatment. This study aimed to evaluate the metal bioaccumulation capacity of autochthonous submerged plants *Potamogeton pusillus* L. specimens exposed to Pb and Cd, individually and combined, over a 3, 7, and 10-day period. The occurrence of uptake enhancement or decrease when both heavy metals were present in the exposure solution was examined. Furthermore, photosynthetic pigments (chlorophylls a, b, and carotenes) and malondialdehyde (MDA) were measured in macrophytes.

Materials and methods

2.1. Reagents and materials

All reagents were of analytical grade. Ultra-pure water was used to prepare standard solutions, dilutions, and blanks. Agilent Technology Multi-Element Calibration Standard Solution (10 mg L^{-1} in 1% nitric acid) was used as a stock solution for the calibration of metal quantification equipment. All glassware used was left in a nitric acid solution overnight and then washed with ultra-pure water. Exposure solutions were prepared using $\text{Pb}(\text{NO}_3)_2$ and $\text{CdCl}_2 \cdot 2\frac{1}{2}\text{H}_2\text{O}$ (Sigma-Aldrich).

2.2. Equipment

Ultra-pure water ($\text{TOC} < 5 \text{ mg L}^{-1}$) was obtained from an Arium 611 UV purification system (Sartorius, Germany). Absorption of chlorophylls, carotenoids, and MDA was recorded using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, USA). Lead and Cd determinations in both abiotic and biotic samples were performed using an Inductively Coupled Plasma Mass Spectrometer (ICP-MS; Agilent Technology 7500 cx Series, California) equipped with an ASX-100 autosampler (CETAC Technologies, Omaha, NE, USA).

2.3. Quality assurance and quality control

The concentrations of the studied elements were determined in triplicate for water and plants. Quality assurance (QA) and quality control (QC) were performed using certified reference materials (CRMs): NIST 1640a (water), NIST 1643e (water), NIST 1547 (peach leaves), and WEPAL 157 (beech leaves). Analysis of these reference materials showed acceptable accuracy for Cd and Pb, with recovery ranging from 95.7 to 105.0% for water, and 82.1 to 105.0% for leaves (Supplementary Material, Table S1). Furthermore, enriched samples were prepared. In order to double the initial concentration of each element, varying amounts of

mixed standard solutions, containing both elements tested, were added to 50 mg of plant sample before the digestion process. The rest of the procedure was the same as that used for non-enriched samples. The average recovery was $79 \pm 6\%$ for Pb and $103 \pm 1\%$ for Cd.

2.4. Plant material and experimental setup

Specimens of *P. pusillus* were collected from a reference site in La Calera, Córdoba, Argentina, between 2019 and 2022 (Monferrán et al. 2012; Griboff et al. 2018). After sampling, the macrophytes were acclimated for one week in a 40-liter glass aquarium located in a greenhouse. The aquarium was filled with tap water, 10% of Hoagland's solution (Hoagland and Arnon 1950), and sediment (1/4) obtained from the sampling site. The acclimation temperature was $21 \pm 1^\circ\text{C}$, and the ratio of light to dark hours was 14:10 (Monferrán et al. 2012). During the exposure experiments, macrophytes were kept in 1.5 L flasks (four plants per container; 7–8 g wet weight per liter) and maintained in 10% Hoagland's solution without sediment.

Four experiments were set up. The first investigated *P. pusillus* removal efficiency and accumulation capacity when exposed to individual Pb and Cd solutions (single-metal system). The exposure concentrations of both heavy metals were 0.5, 1, and 2 mg L^{-1} , used to simulate metal concentrations found in wastewater (Nwosu et al. 2014; Islam et al. 2016; Bokhari et al. 2019). Different exposure times were tested for each treatment, collecting samples on days 3, 7, and 10.

The presence of other metals in the water can alter heavy metal uptake into plant tissue. Hence, in a second experiment, *P. pusillus* removal efficiency and accumulation capacity were examined when both metals, Cd and Pb, were present at the same concentration (0.5, 1.0, and 2 mg L^{-1} ; two-metal system) for 3, 7, and 10 days and at different concentrations. For this last purpose, a set of plants were exposed to 0.5 mg L^{-1} of Pb (or Cd) and increasing concentrations ($0.5, 1, \text{ and } 2 \text{ mg L}^{-1}$) of Cd (or Pb) for 10 days. The removal efficiency and bioaccumulation capacity were analyzed.

A third experimental setup was done to evaluate the metal distribution within the different plant organs (roots, leaves, and stems). Thus, *P. pusillus* was exposed for 10 days to $0.5, 1, \text{ and } 2 \text{ mg L}^{-1}$ Pb or Cd individually. Combined (binary) exposures were also performed using the same concentrations ($0.5, 1.0, \text{ and } 2 \text{ mg L}^{-1}$) of Cd and Pb for 10 days.

Finally, a fourth experimental setup was performed to evaluate the toxicity of Pb and Cd to *P. pusillus*. Thus, the macrophyte was exposed to $0.5, 1.0, \text{ and } 2 \text{ mg L}^{-1}$ single metal solutions for 3, 7, and 10 days. Additionally, plants were exposed to binary solutions of Cd and Pb at the same concentrations ($0.5, 1.0, \text{ and } 2 \text{ mg L}^{-1}$) for 3, 7, and 10 days. Washed plants were then used to measure chlorophyll-a, chlorophyll-b, carotenoids, and MDA as phytotoxic markers.

All experiments had four replicates ($n = 4$; four plants tested at each concentration). Furthermore, there were two types of controls: plant control, consisting of plants grown in 10% Hoagland medium without the addition of metals; and metals control, consisting of metal solutions without plants.

2.5. Metals concentration in exposure solutions

To evaluate changes in the concentration of soluble metals over time, Pb and Cd were determined in the exposure solutions before starting each experiment (t_0) and periodically every two days. Water samples obtained were acidified with ultrapure nitric acid (sub-boiling, Merck), filtered using 0.45 μm nitrocellulose filters (Sartorius, Göttingen, Germany) (Griboff et al. 2018), and stored at 4°C until analysis. The removal efficiency was calculated as follows (Monferrán et al. 2012):

$$Removal (\%) = \left(\frac{C_i - C_f}{C_i} \right) \times 100$$

where C_i and C_f are the initial and residual (final) metal concentrations, respectively.

2.6. Heavy metals content in plants

Macrophytes (whole plant, roots, stems, and leaves) were freeze-dried and then homogenized in a mortar. Subsequently, 50 mg of the sample were digested with 6 mL of ultrapure nitric acid (sub-boiling) and 2 mL of ultrapure hydrochloric acid in closed Teflon tubes on heating plates at 180°C for 8 hours. The obtained samples were filtered using 0.45 μm nitrocellulose membranes and stored at 4°C until analysis (Griboff et al. 2018).

Based on the obtained data, the Concentration Factor (CF) was calculated. This factor is a determinant of the metal uptake capacity of the plant from the aquatic environment, and it can be calculated as the metal concentration in the plant tissue (mg kg^{-1}) with respect to the metal concentration added to water (mg L^{-1}) (Monferrán et al. 2012).

$$CF = \frac{\text{metal concentration in plant tissue}}{\text{metal concentration in water}}$$

2.7. Analysis of physiological parameters contents in plants

Lead and Cd phytotoxicity (single- and two-metal system) was assessed by measuring parameters such as chlorophyll-a (Chl-a), chlorophyll-b (Chl-b), carotenoids, and malondialdehyde (MDA). First, 100 mg of leaves (fresh weight) were ground to dust with liquid N_2 and mixed with 1.5 mL of 80% v/v ethyl alcohol (analytical reagent grade). After that, samples were incubated at 90°C for 20 minutes, left to cool to room temperature, and centrifuged at 14000 rpm for 10 minutes. The chlorophylls, carotenoids, and MDA contents were calculated according to Miranda Pazcel et al. (2018).

2.8. Data Analysis

The results were reported as the mean $\pm$ standard deviation. The data obtained satisfy the assumptions of normality and homogeneity of variances (Shapiro-Wilks and Levene tests used). For the determination of differences between treatments and exposure times, an analysis of variance (ANOVA) using DGC at $p \leq 0.01$ was conducted (Balzarini et al. 2015). Statistical analyses were performed using the InfoStat Version 1.1 software (Di Rienzo et al. 2018).

Results and discussion

2.1. Removal and accumulation of Cd or Pb individually from water solutions

Figures 1A and 1B show the changes in Pb and Cd concentrations in the media of single-metal systems over time. The treatments containing *P. pusillus* show a drop in both metal concentrations from the start of the exposure to day 10. The concentrations of Pb and Cd in the metal control (without plant) do not evidence significant changes throughout the experiment.

From Table 1, it can be noted that *P. pusillus* was able to remove 74–93% and 69–84% of Pb and Cd, respectively when exposed to single metal solutions for 10 days. Removal efficiency decreased as the exposure concentration increased for both metals, having the highest removal (93% and 84%) at the lowest concentration tested (0.5 mg L^{-1}). This decreasing tendency in the Pb and Cd removal efficiencies was reported for other macrophytes, such as *Ceratophyllum demersum*, *Salvinia biloba*, *Lemna minor*, and *Spirodela polyrhiza* (Chaudhuri et al. 2014; Chen et al. 2015; Tello Zevallos et al. 2018).

Table 1
Removal of Pb and Cd (in single- and two-metal system)
from aqueous solutions by *P. pusillus* after 10 days of
exposure.

Initial conc. Pb (mg L ⁻¹)	Initial conc. Cd (mg L ⁻¹)	%Pb Removal	%Cd Removal
0.5	-	93 ± 3	-
1	-	85 ± 5	-
2	-	74 ± 7	-
-	0.5	-	84 ± 5
-	1	-	82 ± 2
-	2	-	69 ± 1
0.5	0.5	94 ± 3	90 ± 4
1	1	94 ± 2	85 ± 3
2	2	92 ± 1	83 ± 1
0.5	0.5	-	82 ± 2
1	0.5	-	85 ± 2
2	0.5	-	84 ± 2
0.5	0.5	86 ± 1	-
0.5	1	91 ± 3	-
0.5	2	90 ± 2	-

Figure 2A shows that *P. pusillus* was able to accumulate Pb in significant amounts with respect to the control group when it was exposed to 0.5, 1, and 2 mg L⁻¹ of Pb. However, the amount of Pb accumulated on days 3, 7, and 10 of exposure showed no significant differences, except on the 2 mg L⁻¹ to 10-day treatment, where the Pb accumulation reached a maximum value of 550 µg g⁻¹ dw. Furthermore, Fig. 2B shows that the Cd accumulation by *P. pusillus* increased significantly as the exposure concentration increased. Also, there is no discernible trend in Cd accumulation over time. The maximum amount of Cd accumulated by the macrophyte was 2045 µg g⁻¹ dw after being exposed to 2 mg L⁻¹ Cd for 7 days.

The ability of aquatic plants to accumulate Pb and/or Cd was also observed in *Myriophyllum aquaticum*, *Egeria densa*, *C. demersum*, *L. gibba*, and *S. polyrhiza* (Chaudhuri et al. 2014; Harguinteguy et al. 2015; Parnian et al. 2016; Dogan et al. 2018). The tendencies observed for the accumulation of Pb and Cd were expected since heavy metal accumulation is described as a time- and concentration-dependent process (Dogan et al. 2018). According to Chojnacka (2010), metal bioaccumulation occurs in two stages: biosorption on the surface of a cell, which can rapidly reach equilibrium; and metabolic processes, which

can transport the bioabsorbed metal into the cell. Furthermore, additional metal ions could be bound to the surface of plants. The accumulation capacity observed for *P. pusillus* could be because this macrophyte is fully submerged, and submerged macrophytes have more metal uptake than the emerged plants due to the surface area of the plant exposed to the contaminated water (Dogan et al. 2018).

Figure 3A shows the bioaccumulation trend for Pb in plants exposed to single metal solutions for 10 days. In this case, a sigmoid-shaped curve is observed, this could be indicating a saturation process either in the uptake mechanisms, the conjugation with endogenous molecules or the internalization to the cytoplasmic vacuoles, among others. Similar results were obtained by Monferrán et al. (2012) in the Cr accumulation pattern when *P. pusillus* was exposed to a binary metal solution (Cr and Cu). Furthermore, Fig. 3B shows the bioaccumulation trend for Cd in plants exposed to single metal solutions for 10 days. As can be observed from this figure, there is a linear relationship between the Cd concentration in the solution and the amount of metal bioaccumulated during 10 days of experiments. Chaudhuri et al. (2014) reported similar findings for Cd removal by *Spirodela polyrhiza* and suggested that Cd accumulation in the plant tissue of this plant increased as a function of initial cadmium concentration in aqueous media.

3.2. Interactions between Pb and Cd: effects on removal efficiency and accumulation capacity

Contaminated waters can contain more than one pollutant, and interactions between them could occur. These interactions can affect plant uptake, removal efficiencies, or phytotoxicity (Alengebawry et al. 2021). However, the underlying mechanisms remain to be understood and could vary among species. The removal efficiencies of *P. pusillus* for the two-metal system are presented in Table 1.

When *P. pusillus* was exposed to a binary metal solution with Pb and Cd at the same concentration, it was able to remove 92–94% and 83–90% of Pb and Cd, respectively. The highest removal efficiencies were achieved at the lowest exposure concentration (0.5 mg L^{-1} Pb or Cd). Nevertheless, when we compare the removal efficiency of single- and two-metal systems, we find that the removal of these pollutants is higher when both are present in the exposure solution at the same concentration.

Figures 2A and 2B show the uptake of Pb and Cd by *P. Pusillus* in the two-metal system. No significant differences were observed in the accumulation of Pb or Cd over time in all concentrations studied. However, significant increases in metal accumulation are observed when exposure concentration increases, with few exceptions. In this system, *P. pusillus* was able to accumulate $1470 \mu\text{g g}^{-1} \text{ dw}$ and $1978 \mu\text{g g}^{-1} \text{ dw}$ of Pb and Cd, respectively, after 10 days of exposure to the 2.0 mg L^{-1} treatment. A comparison between the accumulation capacity of the macrophyte in the single- and two-metal systems shows that *P. pusillus* could accumulate more Pb in the two-metal system. Regarding Cd accumulation, there are no differences for most of the treatments. Although the effect of the combined presence of Cd and Pb on their uptake by aquatic plants is not well understood yet, these results might suggest that, when both metals are

present at the same concentration, the ability of *P. pusillus* to accumulate Pb is affected by the presence of Cd, and the Cd accumulation capacity remains unaltered in the presence of Pb.

The results of the removal efficiency and accumulation capacity of *P. pusillus* when exposed to a fixed concentration of Pb or Cd (0.5 mg L⁻¹) and increasing concentrations of the other metal (0.5, 1, and 2 mg L⁻¹) for 10 days are presented in Table 1 and Figure S1 (Supplementary Material), respectively. Results for Pb show that the removal efficiency and accumulation capacity of *P. pusillus* increased slightly, although not statistically significant. On the other hand, the removal efficiency and accumulation capacity for Cd presented no significant differences.

In the heavy metal uptake process, these pollutants must first pass through the plasma membrane and reach the cytoplasm; this can happen, for example, via ion channels or specialized membrane proteins that mediate ion transport (Sterckeman and Thomine 2020). Generally, submerged macrophytes have thin cuticles, which favor the entry of metal ions to the plants through plasma membrane transporters such as Zinc/Iron Proteins (ZIPs), Natural resistance-associated macrophage proteins (Nramps), Heavy Metals P_{1B}-type ATPases (HMAs), and Cation Diffusion Facilitators (CDFs). In addition, it was demonstrated that metals like Pb and Cd use the Ca²⁺-permeable channels to enter the plants (He et al. 2014; Rodriguez-Hernandez et al. 2015).

Plant cyclic nucleotide-gated channels (CNGCs) are Ca²⁺-permeable channels involved in ion homeostasis. They are also thought to be involved in the uptake of micronutrient ions into cells as well as toxic ions because they lack selectivity in the uptake of different cations (Gobert et al. 2006). Moon et al. (2019) indicated that some CNGCs present in *Arabidopsis thaliana* (AtCNGCs) play a functional role in the Pb²⁺ and Cd²⁺ uptake mechanisms. Nawaz et al. (2019) also demonstrated that eighteen tobacco (*Nicotiana tabacum* L.) CNGC genes, which are phylogenetically related to AtCNGCs, were up-regulated under Cd stress, with gene expression levels 130-fold higher than controls. Given this background, it is reasonable to hypothesize that the presence of Cd, at concentrations of 1.0 or 2.0 mg L⁻¹, induces the expression of different channels in *P. pusillus* involved in Pb uptake. Several studies reported that the presence of one metal influenced the uptake of other metals (Orroño et al. 2012; Redha et al. 2021). To the best of our knowledge, this is the first study of the phytoremediation capacity of *P. pusillus* when Pb and Cd are both present in aqueous solutions. However, reports about this phenomenon in phytoremediation studies involving macrophytes are scarce, and more research is needed to better understand the interaction between heavy metals in solution to propose more efficient remediation strategies by *P. pusillus*.

3. 3. Distribution of Pb and Cd in plant tissues

Table 2 shows the Pb and Cd accumulation patterns in roots, stems, and leaves of *P. pusillus* from single and binary solutions. Our results show that Pb and Cd were distributed throughout the plant regardless of the exposure treatment. In general, the accumulation of Pb and Cd in the vegetal tissues increased as the exposure concentration of each metal in single and binary metal solutions increased. These findings are consistent with previous results from our group showing that Cu, Cr, As, and Hg are directly taken up by roots, stems, and leaves of *P. pusillus* (Monferrán et al. 2012; Griboff et al. 2018).

Table 2

Average concentration of Pb and Cd (from single and binary exposition) in leaves, roots, and stems of *P. pusillus* after 10 days exposures (n = 4). Values are reported as mean $\pm$ SD. Different capital letters indicate significantly different values at a particular part of the plant between concentrations, and different lowercase letters indicate significantly different values at a particular concentration between different plant parts (leaves, stems, and roots) (DGC, $p \leq 0.01$). **(f)**: values refer to Pb concentration in different plant tissues; **(g)**: values refer to Cd concentration in different plant tissues.

		Metal concentration in plants ($\mu\text{g g}^{-1}$ dry weight)			(CF) Concentration Factor		
Initial Pb concentration	Initial Cd concentration	Roots	Stems	Leaves	Roots	Stems	Leaves
(mg L^{-1})	(mg L^{-1})						
0		(1.39 $\pm$ 0.02 ^D) ^a	(0.7 $\pm$ 0.3 ^D) ^a	(0.7 $\pm$ 0.2 ^D) ^a	-	-	-
0.5		(150 $\pm$ 17 ^C) ^a	(65 $\pm$ 16 ^C) ^b	(90 $\pm$ 6 ^C) ^b	300	130	180
1		(294 $\pm$ 50 ^B) ^a	(142 $\pm$ 10 ^B) ^b	(165 $\pm$ 16 ^B) ^b	294	142	165
2		(536 $\pm$ 27 ^A) ^a	(191 $\pm$ 29 ^A) ^c	(323 $\pm$ 7 ^A) ^b	268	96	162
	0	(0.034 $\pm$ 0.003 ^C) ^b	(0.2 $\pm$ 0.1 ^D) ^a	(0.27 $\pm$ 0.04 ^D) ^a	-	-	-
	0.5	(188 $\pm$ 54 ^B) ^a	(133 $\pm$ 11 ^C) ^a	(199 $\pm$ 28 ^C) ^a	376	266	398
	1	(250 $\pm$ 59 ^B) ^a	(233 $\pm$ 31 ^B) ^a	(430 $\pm$ 122 ^B) ^a	250	233	430
	2	(649 $\pm$ 130 ^A) ^b	(466 $\pm$ 31 ^A) ^b	(1040 $\pm$ 64 ^A) ^a	325	233	520
0	0	(3 $\pm$ 2 ^B) ^{a (f)}	(2 $\pm$ 1 ^C) ^{a (f)}	(16 $\pm$ 13 ^C) ^{a (f)}	-	-	-
0.5	0.5	(111 $\pm$ 21 ^A) ^{a (f)}	(53 $\pm$ 3 ^B) ^{a (f)}	(193 $\pm$ 63 ^B) ^{a (f)}	222	106	386
1	1	(229 $\pm$ 189 ^A) ^{a (f)}	(115 $\pm$ 1 ^A) ^{a (f)}	(375 $\pm$ 128 ^A) ^{a (f)}	229	115	375
2	2	(298 $\pm$ 133 ^A) ^{a (f)}	(124 $\pm$ 37 ^A) ^{a (f)}	(535 $\pm$ 127 ^A) ^{a (f)}	149	62	267

		Metal concentration in plants ($\mu\text{g g}^{-1}$ dry weight)			(CF) Concentration Factor		
Initial Pb concentration	Initial Cd concentration	Roots	Stems	Leaves	Roots	Stems	Leaves
(mg L^{-1})	(mg L^{-1})						
0	0	($0.12 \pm 0.04^{\text{C}})^{\text{b}}$ (g)	($0.10 \pm 0.01^{\text{C}})^{\text{b}}$ (g)	($0.34 \pm 0.02^{\text{D}})^{\text{a}}$ (g)	-	-	-
0.5	0.5	($184 \pm 31^{\text{B}})^{\text{a}}$ (g)	($69 \pm 5^{\text{B}})^{\text{b}}$ (g)	($275 \pm 68^{\text{C}})^{\text{a}}$ (g)	368	138	550
1	1	($317 \pm 153^{\text{A}})^{\text{a}}$ (g)	($171 \pm 23^{\text{A}})^{\text{a}}$ (g)	($602 \pm 108^{\text{B}})^{\text{a}}$ (g)	317	171	602
2	2	($509 \pm 208^{\text{A}})^{\text{b}}$ (g)	($202 \pm 13^{\text{A}})^{\text{b}}$ (g)	($982 \pm 60^{\text{A}})^{\text{a}}$ (g)	254	101	491

P. pusillus was able to accumulate higher concentrations of Pb in roots than in leaves and stems when the macrophyte was exposed to 0.5, 1, and 2 mg L^{-1} of Pb. However, the plant exposed to binary solutions did not show Pb preferential accumulation in any tissue (no significant differences were found). Conversely, the leaves showed more Cd accumulation capacity than the stems and roots when exposed to 2 mg L^{-1} of Cd individually and in binary metal solutions. Our results partially agree with those obtained by Fritioff and Greger (2006), who reported that there were no differences between Cd and Pb uptake in root, leaves, and stem of *Potamogeton natans* whether in the presence or absence of other metals in solution.

The Concentration Factor (CF) is a useful parameter for evaluating the ability of plants in accumulating metals with respect to the metal concentration in the surrounding medium (Bokhari et al. 2016). The CFs were calculated for Pb and Cd uptake by *P. pusillus*, under single and binary treatments, and are given in Table 2. CFs were higher for Cd than for Pb throughout different tissues in single and binary metal exposure. In the single-metal system, CFs for Pb were higher in the roots, followed by the leaves and the stems. Previous studies also observed that roots are the primary part of aquatic plants for Pb accumulation (Arán et al. 2017; Castillo Loría et al. 2019). Instead, in the binary solution CFs for Pb were higher in leaves, followed by roots and stems. Nawaz et al. (2019) demonstrated that some CNCG genes, involved in Pb^{+2} uptake, showed increased expression on *N. tabacum* leaves when this plant was exposed to Cd^{2+} . This could be the reason of changes in the trend of Pb CFs in *P. pusillus* when it is exposed to binary metal solution (Pb and Cd). In addition, systems involving exposure to Cd showed higher CF in leaves, then in roots, and finally in stems. This pattern of Cd accumulation was also observed in *Hydrilla verticillate* (He et al. 2016).

Our findings suggest that although all plant parts may accumulate metals, the ability to concentrate metals from the external medium varies between plant parts and metals. A possible reason to explain why

stems of *P. pusillus* accumulated much less Pb and Cd than leaves and roots, either in single and binary solutions, may be because this part of the plant has a transport function through the xylem and phloem by which the different solutes absorbed in the root are transported to the leaves and vice versa (Nas and Ali 2018). Pielichowska and Wierzbicka (2004) suggested that the capability of different tissues to accumulate heavy metals differs depending on many factors, including the anatomical and physiological features of the tissue constituent and its structure, the effectiveness of physiological barriers restricting radial transport of certain metals, physio-chemical properties of metal ions, and the attribution of a plant to be a hyperaccumulator or an excluder. The hypothesis proposed by Monferrán et al. (2012) and Griboff et al. (2018) regarding the chemical and biochemical mechanisms by which *P. pusillus* differentially accumulates metals, such as Cu, Cr, or Hg between root, stem, and leaves, could also be the possible explanation for the results obtained in this work.

3.4. Effect on photosynthetic pigments

Chlorophylls are the main chloroplast pigments responsible for collecting solar radiation during photosynthetic processes (Gomes et al. 2014). It is well documented that the presence of pollutants can alter the content of these pigments in plants and that different metals can affect each component of chlorophyll at different levels (Harguinteguy et al. 2013, 2018; Emiliani et al. 2020). Therefore, it is important to record the changes in Chl *a* and Chl *b* contents when plants are exposed to heavy metals.

When *P. pusillus* was exposed to Pb, Chl *a* content exhibited a concentration and time-dependent behavior, showing an increase as concentration and exposure time to Pb increased, with some exceptions (Table 3). No statistically significant changes are observed in Chl *b* content when *P. pusillus* was exposed to 0.5, 1, and 2 mg L⁻¹ of Pb for 10 days. It has been shown that Pb interferes with and inhibits various physiological processes in plants such as chlorophyll biosynthesis, among others (Orcutt and Nilsen 2000; Cheng 2003). However, our findings are in accordance with others studies that showed that certain amounts of Pb in different plants tissues may have a stimulation effect on chlorophyll content (Manousaki et al. 2009).

Table 3

Content of chlorophyll a, chlorophyll b, and carotenoids in *P. pusillus* exposed at different Pb and Cd concentrations. Means $\pm$ SD are indicated. Different capital letters indicate significant differences between concentrations for the same day and different lowercase letters indicate significant differences between days for the same concentration.

Initial conc. Pb (mg L ⁻¹)	Initial conc. Cd (mg L ⁻¹)	Chlorophyll a ($\mu\text{g g}^{-1}$ fw)			Chlorophyll b ($\mu\text{g g}^{-1}$ fw)			Carotenoids ($\mu\text{g g}^{-1}$ fw)		
		Day 3	Day 7	Day 10	Day 3	Day 7	Day 10	Day 3	Day 7	Day 10
Control	-	(7 $\pm$ 1 ^B) ^a	(27 $\pm$ 7 ^B) ^a	(108 $\pm$ 72 ^B) ^a	(38.4 $\pm$ 0.1 ^B) ^a	(79 $\pm$ 25 ^B) ^a	(142 $\pm$ 48 ^A) ^a	(38.8 $\pm$ 0.3 ^B) ^b	(68 $\pm$ 10 ^B) ^b	(132 $\pm$ 48 ^A) ^a
0.5	-	(43 $\pm$ 8 ^B) ^b	(112 $\pm$ 5 ^A) ^a	(168 $\pm$ 37 ^B) ^a	(105 $\pm$ 18 ^B) ^a	(199 $\pm$ 7 ^A) ^a	(174 $\pm$ 39 ^A) ^a	(68 $\pm$ 23 ^B) ^b	(128 $\pm$ 12 ^A) ^a	(131 $\pm$ 30 ^A) ^a
1	-	(19.4 $\pm$ 0.4 ^B) ^b	(58 $\pm$ 7 ^B) ^b	(304 $\pm$ 40 ^A) ^a	(77 $\pm$ 12 ^B) ^c	(129 $\pm$ 2 ^B) ^b	(230 $\pm$ 7 ^A) ^a	(72 $\pm$ 15 ^B) ^c	(132 $\pm$ 26 ^A) ^b	(205 $\pm$ 37 ^A) ^a
2	-	(176 $\pm$ 81 ^A) ^a	(94 $\pm$ 29 ^A) ^a	(147 $\pm$ 34 ^B) ^a	(244 $\pm$ 41 ^A) ^a	(248 $\pm$ 32 ^A) ^a	(203 $\pm$ 58 ^A) ^a	(172 $\pm$ 78 ^A) ^a	(127 $\pm$ 31 ^A) ^a	(141 $\pm$ 6 ^A) ^a
-	Control	(239 $\pm$ 43 ^A) ^a	(206 $\pm$ 63 ^A) ^a	(245 $\pm$ 70 ^A) ^a	(84 $\pm$ 19 ^B) ^a	(179 $\pm$ 63 ^A) ^a	(175 $\pm$ 39 ^A) ^a	(73 $\pm$ 9 ^A) ^b	(20 $\pm$ 13 ^B) ^c	(125 $\pm$ 11 ^A) ^a
-	0.5	(216 $\pm$ 99 ^A) ^a	(137 $\pm$ 36 ^A) ^a	(54 $\pm$ 8 ^B) ^a	(151 $\pm$ 41 ^A) ^a	(172 $\pm$ 25 ^A) ^a	(123 $\pm$ 3 ^B) ^a	(69 $\pm$ 22 ^A) ^a	(102 $\pm$ 19 ^A) ^a	(97 $\pm$ 4 ^B) ^a
-	1	(161 $\pm$ 18 ^A) ^a	(45 $\pm$ 11 ^B) ^b	(4 $\pm$ 1 ^B) ^b	(58 $\pm$ 5 ^B) ^a	(87 $\pm$ 16 ^B) ^a	(21 $\pm$ 10 ^C) ^b	(64 $\pm$ 7 ^A) ^b	(87 $\pm$ 4 ^A) ^a	(98 $\pm$ 1 ^B) ^a
-	2	(146 $\pm$ 11 ^A) ^a	(29 $\pm$ 1 ^B) ^b	(34 $\pm$ 2 ^B) ^b	(50 $\pm$ 8 ^B) ^b	(59 $\pm$ 8 ^B) ^b	(85 $\pm$ 4 ^B) ^a	(65 $\pm$ 7 ^A) ^a	(26 $\pm$ 2 ^B) ^b	(90 $\pm$ 16 ^B) ^a
Control	Control	(118 $\pm$ 41 ^A) ^a	(302 $\pm$ 223 ^A) ^a	(119 $\pm$ 87 ^A) ^a	(246 $\pm$ 69 ^A) ^a	(235 $\pm$ 4 ^A) ^a	(214 $\pm$ 86 ^A) ^a	(177 $\pm$ 59 ^A) ^a	(294 $\pm$ 74 ^A) ^a	(135 $\pm$ 45 ^A) ^a
0.5	0.5	(224 $\pm$ 11 ^A) ^a	(220 $\pm$ 9 ^A) ^a	(146 $\pm$ 45 ^A) ^b	(190 $\pm$ 13 ^A) ^a	(276 $\pm$ 80 ^A) ^a	(148 $\pm$ 32 ^A) ^a	(177 $\pm$ 8 ^A) ^a	(232 $\pm$ 68 ^A) ^a	(171 $\pm$ 50 ^A) ^a
1	1	(280 $\pm$ 97 ^A) ^a	(262 $\pm$ 87 ^A) ^a	(225 $\pm$ 142 ^A) ^a	(213 $\pm$ 45 ^A) ^a	(224 $\pm$ 18 ^A) ^a	(141 $\pm$ 43 ^A) ^a	(207 $\pm$ 37 ^A) ^a	(227 $\pm$ 40 ^A) ^a	(160 $\pm$ 59 ^A) ^a

2	2	(239 ± 59 ^A) ^a	(237 ± 48 ^A) ^a	(69 ± 14 ^A) ^b	(192 ± 31 ^A) ^a	(250 ± 54 ^A) ^a	(83 ± 7 ^A) ^b	(200 ± 36 ^A) ^a	(228 ± 62 ^A) ^a	(121 ± 5 ^A) ^a
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On the other hand, both parameters (Chl *a* and Chl *b*) tended to decrease as the Cd concentration rose over days in the single metal exposure (Table 3). The drop in Chl *a* and *b* contents occurred after ten days of exposure at 0.5 mg L⁻¹ and after seven days of exposure at 1 and 2 mg L⁻¹ of Cd. Cadmium is one of the principal toxic elements for plants; chlorosis and inhibition of pigment synthesis are common symptoms in aquatic plants exposed to this heavy metal (Piotrowska et al. 2010). The reasons for these symptoms could be disturbances of pigment synthesis mechanisms and Cd-induced nutrient deficiency (Yilmaz and Parlak 2011). Fryzova et al. (2017) also suggested that the ionic imbalance caused by excessive Cd may affect aminolaevulinic acid synthesis (precursor of chlorophyll), increasing the degradation of the pigments. These results suggest that the pigment synthesis mechanism of *P. pusillus* is more sensitive to Cd stress than to Pb.

Conversely, we did not observe significant changes in Chl *a* and *b* relative to the control group when *P. pusillus* was exposed to increasing concentrations of the two-metal system (Table 3), but a significant decrease in both pigments was observed in exposures at 0.5 and 2 mg L⁻¹ of binary solution after 10 days of treatment. Furthermore, the mixture of Cd and Pb exerted less toxicity on photosynthetic pigments in comparison to the exerted by individual Cd solutions. Similar findings were reported by Monferrán et al. (2012), who described antagonist interaction when Cr⁺⁶ and Cu⁺² coexisted in the medium, explaining the lower toxicity, resulting in no changes in Chl *a* relative to the control group in the same macrophyte.

Carotenoids are essential for photosynthesis, acting as secondary pigments and pro-vitamin factors, and are useful to eliminate reactive oxygen species in damaged tissues (Gomes et al. 2014). The carotenoid values of *P. pusillus* under Cd and Pb exposures for 3, 7, and 10 days are presented in Table 3. A significant increase was observed in the carotenoid contents in the aquatic plant when it was exposed to 2 mg L⁻¹ Pb individually for three days ($p < 0.05$). Similar results were obtained after seven days of exposure at all the Pb concentrations tested. However, no statistically significant changes were observed in plants exposed to Pb in single exposure for 10 days at 0.5, 1, and 2 mg L⁻¹.

Carotenes content increased at 0.5 and 1 mg L⁻¹ of Cd on day seven of exposure relative to the control group. Also, a statistically significant decrease at all Cd concentrations evaluated with respect to the control group was found on day ten. Our results agree with what was observed by Emiliani et al. (2020) where the carotenoids concentration in *S. biloba* specimens treated with 10 mg L⁻¹ of Cd showed an increase after 5 days of exposure, followed by a pronounced decrease between days 7 and 10 relative to the control group. Moderate concentrations of Cd caused an increase in carotenoids, which acts as protectors against heavy metal stress. However, the increasing accumulation of Cd throughout the experiment accelerated the degradation of carotenoids in *P. pusillus*.

Finally, there were no significant differences in carotenoid levels when *P. pusillus* was exposed to binary solutions of Cd and Pb between concentrations nor throughout the days of exposure. Our results do not agree with the symptoms of toxicity such as fragility, leaf chlorosis, and root sprouting and twisting, observed in *T. latifolia* treated with binary mixtures of Cd and Pb (Alonso-Castro et al. 2009).

3.5. Effect on malondialdehyde content

Malondialdehyde is one of the main products of lipid peroxidation and can be used to assess membrane damage. In addition, changes in MDA content are generally used to indicate how well an organism resists stress (Pourrut et al. 2013).

An increase in MDA levels is observed as the days of exposure increase when *P. pusillus* is exposed to 0.5 and 1 mg L⁻¹ of Pb (Table 4). However, this same increment is observed in the control group throughout the experimentation days. This suggests that during this assay plants can suffer extra stress. The MDA content was significantly higher with respect to the control in the exposure treatments at the different concentrations of Pb on the third day of the experiment. Additionally, a significant increase in MDA content was observed after seven days of exposure to 0.5 mg L⁻¹ of this metal. Other reports stated in the literature have revealed that Pb can induce oxidative damage in many aquatic plants and the increase in MDA suggests a probable correlation between peroxidation and metal toxicity (Syta et al. 2013; El-Khatib et al. 2014). Moreover, the results show that MDA generally is not affected by increasing concentrations of Cd during the exposure time, except when the plant is exposed to 1 mg L⁻¹ of Cd for 3 days. However, this parameter is impacted by increasing exposure days at 0.5, 1, and 2 mg L⁻¹. Cadmium toxicity was observed to enhance lipid peroxidation in plant cells, which is reflected in higher MDA concentrations (Li et al. 2013).

Table 4
Content of malondialdehyde (MDA) in *P. pusillus* exposed at different Pb and Cd concentrations. Means $\pm$ SD are indicated. Different capital letters indicate significant differences between concentrations for the same day, and different lowercase letters indicate significant differences between days for the same concentration.

Initial conc. Pb (mg L ⁻¹)	Initial conc. Cd (mg L ⁻¹)	MDA (nmol g ⁻¹ dw)		
		Day 3	Day 7	Day 10
Control	-	(16 $\pm$ 5 ^B) ^b	(38 $\pm$ 2 ^B) ^a	(50 $\pm$ 10 ^A) ^a
0.5	-	(27 $\pm$ 3 ^A) ^b	(58 $\pm$ 3 ^A) ^a	(50 $\pm$ 10 ^A) ^a
1	-	(33 $\pm$ 4 ^A) ^b	(41 $\pm$ 2 ^B) ^b	(50 $\pm$ 10 ^A) ^a
2	-	(30 $\pm$ 10 ^A) ^a	(40 $\pm$ 10 ^B) ^a	(40 $\pm$ 10 ^A) ^a
-	Control	(25 $\pm$ 1 ^A) ^a	(50 $\pm$ 20 ^A) ^a	(50 $\pm$ 20 ^A) ^a
-	0.5	(30 $\pm$ 10 ^A) ^b	(40 $\pm$ 10 ^A) ^a	(40 $\pm$ 10 ^A) ^a
-	1	(16 $\pm$ 1 ^B) ^b	(32 $\pm$ 4 ^A) ^a	(32 $\pm$ 3 ^A) ^a
-	2	(23 $\pm$ 4 ^A) ^b	(32 $\pm$ 1 ^A) ^a	(34 $\pm$ 1 ^A) ^a
Control	Control	(68 $\pm$ 1 ^A) ^a	(48.3 $\pm$ 0.2 ^A) ^a	(30 $\pm$ 10 ^A) ^a
0.5	0.5	49 $\pm$ 2 ^B) ^b	(63 $\pm$ 4 ^A) ^a	(35 $\pm$ 2 ^A) ^c
1	1	(43 $\pm$ 2 ^C) ^b	(50 $\pm$ 10 ^A) ^a	(36 $\pm$ 1 ^A) ^b
2	2	(42 $\pm$ 3 ^C) ^a	(50 $\pm$ 10 ^A) ^a	(25 $\pm$ 1 ^A) ^b

No significant changes were observed in any of the concentrations evaluated during 7 and 10 days of binary-metal exposure, although a significant drop in MDA content was observed in plants exposed to binary-metal solution with respect to the control group after three days of exposure. This tendency was also observed from days 3 to 10 when the macrophyte was exposed to 0.5 and 2 mg L⁻¹. Decreased MDA content can be considered as an indication of low peroxidation of lipids, which can be caused by increased activity of the antioxidant system (Ghuge et al. 2023). Plants have strong antioxidant systems, which can protect biomembranes from oxidative damage by lipid peroxidation.

Conclusions

To the best of our knowledge, there is no research regarding the removal efficiency, accumulation, and tolerance of Pb and Cd, either individually or combined, by *P. pusillus*. Furthermore, there is limited information about the concentration and time-dependent effects of Pb and Cd on *P. pusillus* physiological parameters.

In this study, we observed a high correlation between the final Pb and Cd concentrations in water and the heavy metal contents in the macrophyte, whether in the single- or two-metal system. In addition, exposure to the two-metal system, containing 1 and 2 mg L⁻¹ of Cd and Pb, resulted in enhanced absorption of Pb without affecting Cd accumulation in comparison to individual exposure. Regardless of the metal concentration used, it was shown that both Pb and Cd are distributed throughout the plant, including roots, stems, and leaves, suggesting that these metals are directly taken up from the water by the leaves and roots.

Considering the accumulation capacity and removal efficiency of Pb and Cd by this macrophyte, and that the combined presence of both metals in aqueous solutions decreases some toxic effects, *P. pusillus* can effectively be proposed for the removal of these heavy metals from polluted aquifers. Therefore, the results of this work highlight the importance of performing further, perhaps longer, metal exposure tests to identify additional physiological changes that might function as a tool for the selection of macrophytes for remediation purposes. Further studies are also required to understand the interaction between both metals and this plant leading to the enhanced absorption of Pb in the presence of Cd by *P. pusillus*.

Declarations

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Ethical Approval

Not applicable.

Consent to Participate

Not applicable.

Consent to Publish

Not applicable.

Author contributions

Rivela CB: conceptualization, investigation; methodology, formal analysis, original draft writing, review and editing, visualization.

Griboff J: methodology, formal analysis, validation, writing, and editing.

Arán DS: methodology, formal analysis, validation, writing, and editing.

Cortés FL: formal analysis, validation, writing, and editing.

Valdés ME: formal analysis, validation, writing, and editing.

Harguinteguy CA: formal analysis, validation, writing, and editing.

Monferran MV: conceptualization, investigation; funding acquisition, resources, supervision, project administration, original draft writing, review and editing, visualization.

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Competing Interests

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

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