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Phytostabilization of metal(loid)s by ten emergent macrophytes following a 90-day exposure to industrially contaminated groundwater

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A B S T R A C T

Better understanding of macrophyte tolerance under long exposure times in real environmental matrices is crucial for phytoremediation and phytoattenuation strategies for aquatic systems. The metal(loid) attenuation ability of 10 emergent macrophyte species (*Carex riparia*, *Cyperus longus*, *Cyperus rotundus*, *Iris pseudacorus*, *Juncus effusus*, *Lythrum salicaria*, *Menta aquatica*, *Phragmites australis*, *Scirpus holoschoenus*, and *Typha angustifolia*) was investigated using real groundwater from an industrial site, over a 90-day exposure period. A “phytobial” treatment was included, with 3 plant growth-promoting rhizobacterial strains. Plants exposed to the polluted water generally showed similar or reduced aerial biomass compared to the controls, except for *C. riparia.* This species, along with *M. aquatica,* exhibited improved biomass after bioaugmentation. Phytoremediation mechanisms accounted for more than 60% of As, Cd, Cu, Ni, and Pb removal, whilst abiotic mechanisms contributed to ~80% removal of Fe and Zn. Concentrations of metal(loid)s in the roots were generally between 10–100 times higher than in the aerial parts. The macrophytes in this work can be considered “underground attenuators”, more appropriate for rhizostabilization strategies, especially *L. salicaria, M. aquatica, S. holoschoenus,* and *T. angustifolia*. For *I. pseudacorus, C. longus*, and *C. riparia;* harvesting the aerial parts could be a complementary phytoextraction approach to further remove Pb and Zn. Of all the plants, *S. holoschoenus* showed the best balance between biomass production and uptake of multiple metal(loid)s. Results also suggest that multiple phytostrategies may be possible for the same plant depending on the final remedial aim. Phytobial approaches need to be further assessed for each macrophyte species.

Introduction

Environmental contamination by metal(loid)s is a global problem associated with anthropogenic activities, contaminating pristine soil and water [1]. In the last 20 years, legislation has been developed to help reduce metal(loid)s in products, remove them from waste and remediate

Abbreviations: BCF, Bioconcentration Factor; BPW, Polluted Water + Plant Growth Promoting Rhizobacteria (PGPR) “RBM” Mixture; CB, Control Rhizobacteria Mixture; CW, Control Water; DO, Dissolved Oxygen; DT, Decision Tree; DW, Dry Weight; EC, Electrical Conductivity; FTW, Floating Treatment Wetland; PGPR, Plant Growth Promoting Rhizobacteria; MFS, Microcosms Floating System; FW, Fresh Weight; ICP-MS/MS, Inductively Coupled Plasma-Mass Spectrometry triple quadrupole; ICP-OES, Inductively Coupled Plasma-Optical Emission Spectrometry; ORP, Redox Potential; PW, Polluted Water; RBM, Rhizobacteria Mixture; TDS, Total Dissolved Solids; TF, Transference Factor.

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existing contamination [2]. Phytoremediation offers affordable and eco-friendly solutions for treatment of soil and water contamination. By leveraging plants' natural capabilities, these methods absorb and accumulate pollutants, addressing diverse sources of metal(loid)s and organic contaminants [3]. Selected plants must show tolerance to elevated pollutant concentrations, fast growth, and reasonable availability, as these properties vary with reference to plant and the type of contamination [4,5]. Aquatic phytoremediation uses emergent macrophytes for the removal and degradation of pollutants, that grow in the water bodies margins, either in submerged and emergent condition, allowing both the contaminant removal from the water column and the rooting substrate [4]. Castillo Loría et al. [6] assessed the toxicity of Pb in roots and aerial parts of submerged *Salvinia biloba* and suggested the use of this water fern for management of residual water bodies contaminated with Pb. Schück and Greger [4] screened the capacity of 34 wetland plant species to remove metals (Cd, Cu, Pb, and Zn) in water, and identified that *Carex pseudocyperus* and *Carex riparia* were the most efficient and versatile.

The accumulation of metal(loid)s in the plant is largely influenced by the metal(loid) phytoavailability, the rate of absorption by the roots, and the translocation from roots to aerial tissues [7]. Most species utilize root bioactivation mechanisms to enhance root absorption (rhizosphere acidification, secretion of organic acids, metal chelation, or enzyme production to increase available nutrients). The main limitation of macrophyte metal(loid) uptake is the toxicity that the target pollutant can cause. However, detoxification mechanisms allow species to avoid the negative effects of the metal(loid)s; for example, more than 50% of the Ca, Cd, Co, Fe, Mg, Mn, and Zn recovered in the roots of *Pistia stratiotes* was attached to the external root surfaces indicating the ability of the plant to exclude metal(loid)s and, thus, maintain tolerable levels internally [8]. Newete and Byrne [9] also stated that the extent of the root system affects the ability of macrophytes to remove metal pollutants, with fibrous root systems being superior to taproot, due to their larger surface area. Environmental factors that are also important for the uptake and accumulation of metal(loid)s include temperature, light, pH, and salinity [10].

Plant growth-promoting rhizobacteria (PGPR) and mycorrhizal fungi obtain C-sources for their metabolism from the plant during "phytobial" partnerships; in return, they promote plant growth, decrease metal(loid)s toxicity, and/or improve the biodegradation of persistent organic compounds [11]. The application of PGPR can enhance the growth of hyperaccumulator plants by improving their metal(loid) availability, tolerance, and accumulating capacity. PGPR can produce metabolites which aid in the solubilization and provision of essential nutrients to the plant, alleviating stress at the same time [12]. Four macrophytes increased their removal capacity for five trace metals (Fe, Mn, Ni, Pb, and Cr) in bacterially assisted Floating Treatment Wetlands (FTWs) for the clean-up of artificially spiked river water [13]. In FTWs using *Phragmites australis* bioaugmented with several bacterial strains, heavy metals were successfully removed [14]. *P. australis* also removed phenol from water more efficiently in combination with three bacterial strains [15]. Some studies suggest that plant-associated endophytes may offer more potential for phytobial remediation than plant-associated rhizosphere bacteria. The use of endophytes native to the host plant reduces competition between bacterial strains, avoiding needs for reinoculation [16,17].

While multiple studies have assessed the potential of PGPR and mycorrhizal fungi to improve plant performance for agronomical applications, studies for phytoremediation approaches, particularly in aquatic systems, are scarce. In the present study, we hypothesized that macrophytes are metal(loid) tolerant plants that can be used to attenuate mixed metal(loid) contamination in polluted water. We also hypothesized that a phytobial strategy (bioaugmentation of plants with PGPR) can improve the metal(loid) removal efficiency and growth of wetland macrophytes. To test these hypotheses, 10 emergent aquatic species were exposed for 90 days to groundwater industrially contaminated by 7 different metal(loid)s in the presence or absence of PGPR (three strains). Plant incubations were performed in microcosm floating systems (MFS) in the greenhouse. Plant growth was evaluated by means of aerial biomass production. For assessment of metal(loid)s, removal concentrations in groundwater as well as concentrations in aerial parts and roots were measured. The contribution to contaminant removal by abiotic or phytoremediation mechanisms was estimated for each metal(loid) and plant.

Materials and methods

Experimental setup

Plantlets from 10 different species of emergent macrophytes were provided by "Viveros La Dehesa" (Valdeobispo, Cáceres, Spain, https://www.viverosladehesa.com/). The species were *Carex riparia*, *Cyperus longus, Cyperus rotundus, Iris pseudacorus*, *Juncus effusus*, *Mentha aquatica, Lythrum salicaria*, *Phragmites australis*, *Scirpus holoschoenus*, and *Typha angustifolia*. Strict quality control measures to ensure the genetic consistency of plant stock were maintained for plant propagation. Macrophytes of uniform genetic origin were bought that were produced predominantly by propagating vegetative rhizomes, turions, or stolons. Plants were acclimated under controlled greenhouse conditions for two months. Initially 4 morphologically similar plants per 4 L bucket were used, however, after the one month of acclimation, plants that were showing uniform growth were selected, while the other plants that were either showing lower or higher growth were removed. The experiment was conducted using microcosms floating systems (MFS) consisting of 4 L buckets with a floating system of extruded polystyrene holding plastic baskets, open at the bottom to allow root development; other hole was used for water sampling and monitoring (Suppl. Fig. S1). The borders of the pots were covered with aluminium foil to avoid algae proliferation. Plants were distributed randomly and conveniently rotated to avoid preferred positions of temperature and light. MFS' water level was steadily monitored and kept adequate to avoid changes in media concentration. (Suppl. Fig. S1).

After the acclimatization period, 4 treatments were applied in triplicate MFS, for each of the 10-aquatic species. A total number of 132 MFS were introduced (Suppl. Table S1):

- Control (CW): Tap water + Hoagland's solution.
- Polluted Water (PW): Tap water + Hoagland's solution + real polluted groundwater.
- Polluted Water + Plant Growth Promoting Rhizobacteria (PGPR) "RBM" Mixture (BPW): Tap water + Hoagland's solution + PGPR mixture + real polluted water.
- Control Rhizobacteria Mixture (CB): Tap water + Hoagland's solution + PGPR Mixture.

The composition of Hoagland solution used was 5 mM KNO_3, 5 mM $Ca(NO_3)_2.4\ H_2O$, 67 mM Fe-EDTA, 2 mM $MgSO_4.7\ H_2O$, 1 mM NH_4NO_3, and 0.5 mM KH_2PO_4, for macronutrients; the trace element concentrations were: 50 μM H_3BO_3, 10 μM $MnCl_2.4\ H_2O$, 1 μM $ZnSO_4.7\ H_2O$, 0.3 μM $CuSO_4$, and 0.5 μM $Na_2MoO_4.2\ H_2O$. Unvegetated control buckets, with the same composition as those vegetated for each of the 4 treatments, allowed to evaluate the processes involved in metal(loid)s removal by abiotic deposition. Each MFS, according to the correspondent treatment, had the following composition: 200 mL concentrated Hoaglands solution (18x dilution); 200 mL PW (18x dilution); 10 mL PGPR mixture, namely "RBM" (Rhizobacteria Mixture, 1:1:1; see below for details). In the case of treatments with inoculation of PGPR (+RBM), these were inoculated before spiking the real polluted water into the correspondent MFS, to allow microorganisms to properly colonize the rhizosphere and/or endophyte the plant along several weeks.

Monitoring and sampling scheme

The phytoremediation experiment lasted 90 days and consisted of the following monitoring program:

i. Water parameters: pH and electrical conductivity (EC), among others, at regular fortnightly intervals; and metal(loid)s concentrations (details provided in the sections of Physical-chemical determinations in the rhizosphere water, and Metal content determinations via ICP-OES and ICP-MS/MS in water and plant tissues);
ii. Plant parameters, including plant biomass of roots and shoots/leaves (aerial), at 90 days (end-point); metal(loid)s concentrations in both compartments; and bioconcentration and transference factors (details provided in the section of Metal content determinations via ICP-OES and ICP-MS/MS in water and plant tissues).

Inoculation and survival of PGPR

The metal(loid)-tolerant RBM (1:1:1) mixture of 3 PGPR strains (IR9, *Enterobacter* sp.; IR29, *Enterobacter* sp.; and IR39, *Pseudomonas* sp.), previously isolated and characterized by the Institute of Technology Carlow (unpublished results), was inoculated to the corresponding treatments (CB, BPW) before the onset of the phytoremediation experiment. All the strains were individually cultured in modified nutrient medium (0.5% peptone, 0.3% yeast extract, 0.3% NaCl) at 30 °C overnight. After this incubation, cells were harvested by centrifugation (20 min at 1800 *g*), washed once with Dulbecco's phosphate-buffered saline (DPBS) and re-suspended in Hoagland medium. The optical density was adjusted to $OD_{600} = 0.07$ ($\approx 10^8$ CFU mL^{-1}). Bacterial suspensions were mixed in 1:1:1 ratio and 10 mL of the resulting consortia was inoculated in each MFS (at a final concentration of 2.5 mL L^{-1}), where required.

For the survival analysis of the inoculated PGPR in the rhizosphere in the corresponding microcosms, rhizomes and other endophytic tissues were harvested and analysed. Briefly, culture-dependent plate-counting method was used, both from directly collected rhizosphere water aliquots and plant tissues, including aerial and rhizosphere plant parts. Negative controls were included in every case. The water samples were directly plated onto nutrient agar plates, at several dilutions. For roots/rhizomes, two distinct procedures were followed: to detect the presence of endophytic bacteria, plant tissues were surface sterilized with 70% EtOH, followed by their grinding with 0.9% NaCl; for analysing the presence of rhizosphere bacteria, sterilization was omitted. The lysates of grounded material were plated onto nutrient agar plates. Plates were incubated at 30 °C for 24–48 h. In those samples with observed positive bacterial growth, 10 randomly selected colonies were plated onto 1/10 strength nutrient agar plates, containing 6 mM $NaAsO_2$, and were incubated at 30 ºC for up to 5 days, including R9, R29, and R39 strains, and *Escherichia coli*, as positive and negative controls, respectively.

Physiological parameters

A weekly follow-up of the phenotypes and performance of the plants was performed. Fresh and dry biomass of aerial parts and roots was quantified gravimetrically at 90 days and expressed in g±g. The samples of fresh biomass were examined shortly after harvesting. For aerial biomass plant were dissected just above the growth substrate, while for root biomass, the growth substrate was carefully removed, and the plant roots were washed with distilled water to eliminate any possibly surface related particles. The roots were then air dried and used for fresh weight quantification. The dried biomass (root and shoot) was quantified after dehydrating the fresh biomass in an oven operating at 60 °C for 96 h, however, when necessary, the dehydration was carried until it reached a consistent weight.

Physical-chemical determinations in the rhizosphere water

The physical-chemical properties of the rhizosphere water were monitored fortnightly using a multiparameter probe (model HI98194, HANNA). This allows the simultaneous determination of pH and electrical conductivity (EC), redox potential (ORP), dissolved oxygen (DO), and total dissolved solids (TDS). Briefly, the multiparametric system was first calibrated, every time before the measurement were taken, using the company provided calibration standard solution (HI9828, Multiparameter Quick Calibration Solution HANNA). Electrode was rinsed by distilled water between the measurement of different parameters in each mesocosms.

Metal content determinations via ICP-OES and ICP-MS/MS in water and plant tissues.

Major and trace elements in all water and plant samples were determined by ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometry, SPECTRO GENESIS, AMETEK, Germany), and/or ICP-MS/MS (8900 Inductively Coupled Plasma-Mass Spectrometry triple quadruple, Agilent, USA), respectively, depending on the metal(liod)s level in groundwater. Water samples were filtered at 0.22 µm and acidified with 1 mL o concentrated HNO_3 in a 1:10 dilution ratio. When necessary, the relevant dilutions were prepared for the analysis, if the concentration were higher than the linear response limit. Dried plant tissue samples (0.25 - 0.5 g) were weighed on a precision balance (± 0.1 mg), mixed with 2 mL of H_2O_2 (33% v/v) and 8 mL of concentrated nitric acid (65% w/v) and digested in a microwave oven (ETHOS ONE, Milestone, USA). After the digestion process (30 min at 180 °C), the liquid was filtered (CF/WASH110 filter paper, Ø 110 mm, Scharlau), and adjusted to 25 mL using MilliQ water. All material used were previously washed with diluted HNO_3 and rinsed with MilliQ water. To maintain the track precision a multi-standard of 21 elements of known concentration (ICP multi-element standard solution - 89166.180, VWR, Germany) and blanks were also included (one for each 15 samples). In each batch of digestion samples, a certified plant material standard was introduced (ERM-CD281 Rye grass certified material) to track accuracy. All the sample values were corrected with the respective to the values found in blank. When necessary, the relevant dilutions were prepared for the analysis, if the concentration was higher than the standard detectable limit. The certified value for the metal(liod)s in stranded reference material and their recovery is presented in Supplementary Table S2. The accuracy of instrument measurement ranged between highest for Zn with a recovery of 99.01 ± 3.28, while lowest for As 94.129 ± 1.23 relative to the standard certified reference material used (ERM-CD281 Rye grass certified material) along with errors of measure less than ± 5. The average percentage recovery for all metal(liod) was identified to be 95.56%, with a percentage RSD of 2.68%. This indicates that the analytical method used to measure the metal concentrations is accurate and reproducible.

Metal(loid)s uptake were also enumerated using bioconcentration factor (BCF) and translocation factor (TF) [17]. The BCF is described as the capacity of plants for the elemental accumulation into the roots compartment with respect to a substrate, while the TF is calculated from the concentration of the element in the aerial part of the plant compared to its concentration in the roots [28].

$$\mathrm{BCF} = \frac{Metal(loid)\ in\ \ root}{Metal(loid)\ \ content\ \ in\ \ growth\ \ substrate}$$

$$\mathrm{TF} = \frac{Metal(loid)\ in\ \ shoot}{Metal(loid)\ \ content\ \ in\ \ roots}$$

Decision tree preparation

The basic idea behind decision trees (DTs) is to create a model that predicts the value of a target variable based on several input variables. Scikit-learn is a Python module integrating a wide range of state-of-theart machine learning algorithms such is Classification And Regression Tree (CART). The CART algorithm generates only binary trees, where nonleafy nodes always have two branches (i.e., questions only have yes/no answers). This algorithm divides the main dataset into subsets using a single feature and a threshold criterion, and the Gini impurity index is used to calculate the probability of incorrectly classifying a feature. The process is repeated until the instance reaches a leaf node (a node that does not result in a division of the dataset during the tree's construction). Gini impurity index (G) is used to calculate the amount of probability of a specific feature that is classified incorrectly when selected randomly:

$$G = \sum_{i=1}^{C} p_i \times (1 - p_i)$$

Where, C is the total number of classes and p_i is the probability of picking the data point with the class i.

In this work, a dataset composed of metal concentrations measured in roots and aerial parts (14 variables in total), as well as the variable "plant species", was used for classification. The data set has 120 instances ($N = 120$), obtained as follows: 3 biological replicates/species, 4 treatments and 10 species. The parameters selected followed Gini as criterion, maximum depth of 5, whereas the others were left by default. The visualization of the data in the classification trees was made with a python library for decision tree visualization and model interpretation (https://github.com/parrt/dtreeviz). The performance of the model is evaluated by a 10-fold cross validation [18,19].

Statistical analyses

Results are the mean of at least three independent biological replicates. One-way ANOVA was performed after checking for normality and homogeneity of variance assumptions. Significant differences between means were assessed by the Tukey' (HSD, Honestly-Significant-Difference) *post-hoc* test, with a significance of $p < 0.05$. For mean comparison of metal(loid)s between PW and BPW treatments, a *t-test* was applied. The statistical package IBM SPSS Statistics v.22 was used.

Results

Aerial biomass

The results regarding the plant aerial fresh biomass after 90 days of exposure to the contaminated groundwater revealed differences among treatments within each individual plant species. For most of the studied species, exposure to contaminated groundwater (i.e., PW/BPW) was either detrimental or had no effect at the end of the incubation (Fig. 1a for aerial biomass and Fig. 1b for root biomass). Significant differences among the four treatments (CW, PW, CB, and BPW) were observed for *C. riparia*, *J. effusus*, *M. aquatica*, *L. salicaria*, *P. australis*, and *T. angustifolia*. Interestingly, aerial biomass for *C. riparia* was significantly greater when exposed to the polluted water (PW) compared to the control (CW). Noteworthy, for this species, the fresh aerial biomass was significantly larger in the polluted bioaugmented treatment (BPW) than the respective control (CB) and the polluted water treatment (PW). The largest aerial biomass of all treatments was measured for the BPW grown *C. riparia* plants, suggesting a positive effect of PGPR′on this aquatic macrophyte. For *J. effusus* and *M. aquatica* aerial biomass was significantly lower for PW compared to CW; this was not observed for BPW, suggesting a beneficial effect of the rhizobacteria bioaugmentation. In the case of *L. salicaria*, plants exposed to polluted water (PW and BPW)

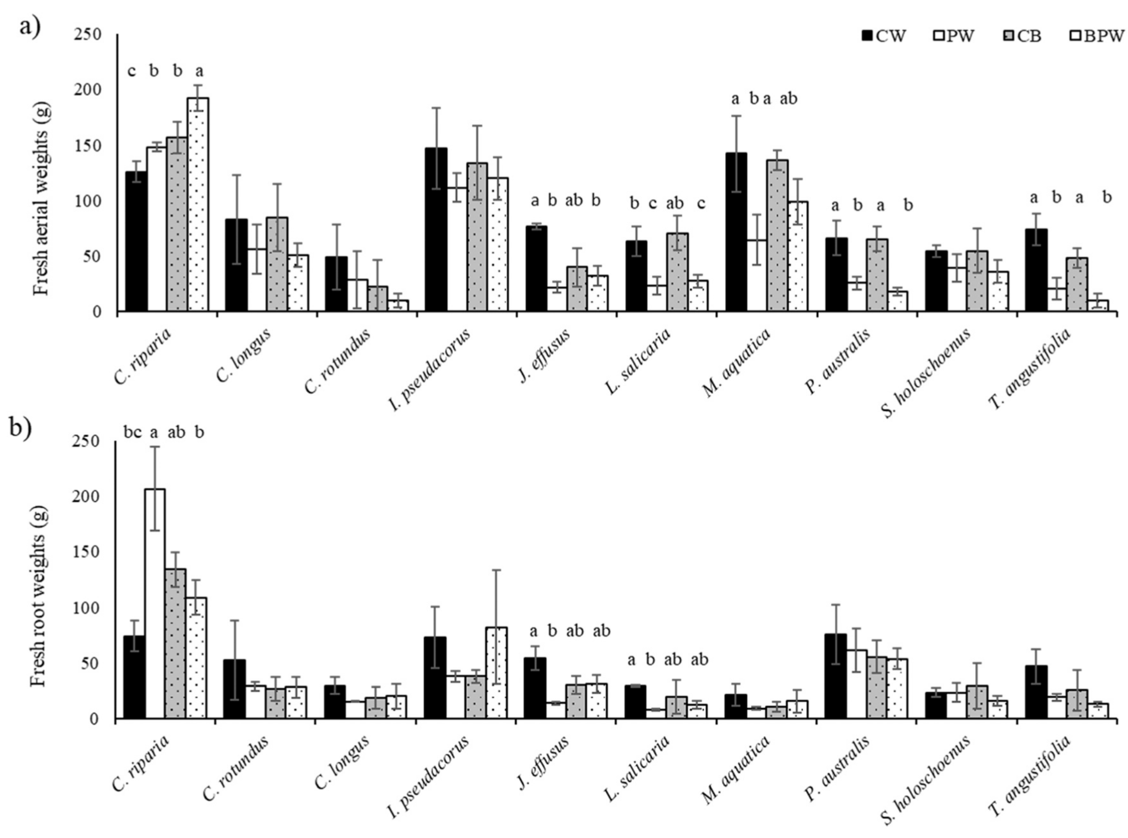

Fig. 1. Fresh Biomass [a) for aerial, and b) for root] produced by the ten-aquatic species under study after 90 days of exposure to the correspondent treatment. Data (vertical bars) represent the mean of three biological replicates (± standard deviation, SD). Different letters indicate significant differences between treatments for each plant species ($P < 0.05$, One-Way ANOVA followed by Tukeýs post hoc test). CW: control water; PW: polluted water; CB: Control bacteria; and BPW: bacteria + polluted water; FW. Fresh Weight in grams (g).

showed lower biomass than the controls. A similar pattern was observed for *P. australis* and *T. angustifolia*. Finally, for *C. longus*, *C. rotundus*, *I. pseudacorus*, and *S. holoschoenus*, no significant differences were found between the four treatments. For the root biomass variant response of biomass production was observed, notably significant differences between treatments for *C. riparia, J. effusus,* and *L. salicaria,* was noted, while no statically significant differences were observed for other plant. In the case of *C. riparia* plant in PW showed significantly highest levels, while for *J. effusus* and *L. salicaria* highest root biomass was noted for CW.

Physical-chemical parameters

Changes in pH and EC of polluted water are shown in Suppl. Figs. S2 and S3. The results displayed correspond to the data measured in the rhizosphere water fortnightly after 0, 15, 30 and 45 days of exposure. Monitoring was finalized on day 45 corresponding to time 3 (T3) since pH and EC were stabilized. Although results for pH showed a great variability, some general patterns were observed (Suppl. Fig. S2). As a rule, the initial pH of the polluted water treatments (PW/BPW) was 1–1.5 units lower than the respective controls (CW/CB). This can be attributed to the acidic nature of the polluted water. As time progressed, the pH values of the water increased significantly for the polluted treatments (PW/BPW) reaching comparable levels to those of the controls (CW/CB). In general, pH values ranged between approx. 5.5–6.5 for most treatments and monitoring times. The trend was towards neutralization from initial acid pH values, in most of the tested species, except for *I. pseudacorus* and, to a lesser extent, for *L. salicaria*, *P. australis,* and *S. holoschoenus*. In those species, a steady basification was detected in the PW treatment. In contrast to the general neutral pH tendency in the PW, those MFS corresponding to BPW treatment tended towards basification. *I. pseudacorus* exhibited the lowest pH values already at T0 in PW and BPW, to later increase with time; whereas in CW and CB the trend was a decreased pH at T1 in comparison with T0, to later raise again, especially at T3. For EC significant differences were observed between treatments for the same species and between exposure times for the same treatment (Suppl. Fig. S3). As a rule, EC was approx. 2–3 times greater for treatments PW and BPW than their respective controls for most species, because of the high EC of the original polluted groundwater sample. Generally, the EC range for the polluted treatments PW and BPW ranged approx. between 400–800 μS cm^{-1}, and for the controls (CW and CB) between 200–600 μS cm^{-1}. For various plant species including *C. longus*, *I. pseudacorus*, *L. salicaria*, *P. australis*, and *T. angustifolia*, the EC gradually decreased after an initial increase at T1 for the PW and BPW treatments. Likewise, the multiparametric probe was used for the measurement of other parameters including dissolved oxygen (DO) and redox potential (ORP) (data not shown). The DO values ranged between 2.4 to 5.2 mg L^{-1} at T0 and gradually decreased to 2.15 to 3.5 mg L^{-1} at T3, indicating O_2 consumption in the rhizosphere water. The redox potential (ORP) ranged between 150 and 400 mV and, thus, is representative of oxidic conditions.

Metal(loid) patterns in water, aerial, and root compartments

The contaminated groundwater collected from an industrial site in Belgium used in this present study was characterized by high concentrations (above permissible levels) of metal(loid)s (Table 1), low pH (~3.7), and high electrical conductivity (~5320 μS/cm). Further, the changes in metal(loid) concentrations in water were monitored for the PW and BPW treatments every 15 days (T1 = 15 days, T2 = 30 days, and T3 = 45 days). For both treatments a significant metal(loid) removal (≥70%) was achieved already at T1 (15 days) for most plant species. At T3 (45 days) metal(loid) removal was practically complete (95–100%). For instance, in the case of Zn and Cu for the PW treatment (Suppl. Fig. S4, and Suppl. Table S3), the initial concentration values rapidly decreased by > 99% for Cu and > 90% for Zn in the presence of *C. riparia* and *P. australis* at T1. The removal of Zn was steadier in the presence of *I. pseudacorus* (69% at T1) and *M. aquatica* (60% at T1) compared to other plant species. A similar pattern was observed for Cu removal in the presence of *C. rotundus* (45% at T1) and *M. aquatica* (68% at T1). In general, removal efficiency was similar between the bioaugmented (BPW) and non-bioaugmented (PW) treatments. For *C. rotundus* and *M. aquatica* the BPW showed significantly greater Cu removal at T1 than PW.

Table 1
Initial concentrations for the target elements in the original groundwater sample as determined by ICP-OES / ICP-MS. Results are expressed in mg L^{-1}. Metals: Cadmium (Cd), Copper (Cu), Iron (Fe), Nickel (Ni), Lead (Pb), Zinc (Zn); metalloids: Arsenic (As).

Metal(loid)	mg L^{-1}
As	0.450 – 0.500
Cd	2.000 – 2.500
Cu	160 – 1000
Fe	250 – 400
Ni	130 – 150
Pb	0.200 – 0.350
Zn	70 – 320

Metal(loid)s concentrations in both roots and aerial compartments exhibited a great variability at the end of the experiment (90 days) for both treatments (PW and BPW) (Fig. 2). Nevertheless, for most plant species a general trend was observed with higher accumulation of metal (loid)s (As, Cd, Cu, Ni, Pb, and Zn) in the roots (10 to 100 times) in comparison to the aerial parts. This was particularly evident for root concentrations of Fe (range from 200–10000 μg Kg^{-1} DW), Cu (200–5000 μg Kg^{-1} DW), Zn (100–1500 μg Kg^{-1} DW) and Ni (50–1000 μg Kg^{-1} DW). Concentrations for As, Cd and Pb in the roots were between 1–20 μg kg^{-1} DW. In the aerial compartment, metal(loid) s concentrations ranged from 10–200 μg kg^{-1} DW for Cu, Fe, Ni and Zn, and between 0.01–2 μg kg^{-1} DW for As, Cd and Pb. Metal(loid) uptake also differed depending on the species considered. Greater metal(loid) concentrations for both roots and aerial parts were generally found for *L. salicaria* followed by *M. aquatica*, *J. effusus,* and *S. holoschoenus* compared to other species. Differences in metal(loid) uptake between the bioaugmented (BPW) and non-bioaugmented (PW) treatments were also found for some species. An increased uptake in the presence of PGPR (BPW) was observed for the following metal(loid)s and plants: As in the aerial of *L. salicaria*, *M. aquatica*, and *P. australis*; Cd in the aerial of *J. effusus* and *S. holoschoenus*; Cu, Fe and Ni in the aerial of *M. aquatica*; Zn in the roots of *M. aquatica* and *S. holoschoenus* and in the aerial *of M. aquatica*. In contrast, a reduced uptake in the presence of PGPR (BPW) was observed for the following metal(loid)s and plants: Cu in the roots of *L. salicaria*; Pb in the roots of *J. effusus* and *T. angustifolia* as well as in the aerial compartments of I. *pseudacorus*, *M. aquatica*, and *P. australis*. To further understand the distribution and the predominance of the targeted metallic elements in the roots and aerial parts of the tested species in the PW treatment, a classification using a decision tree methodology was prepared (Suppl. Fig. S5). The prediction results in the form of proportion values at the terminal nodes are the species classified with the highest concentration of Fe and Cu in the leaves, like *M. aquatica*, and also Fe in the roots, like *L. salicaria.*

To assess the contribution of phytoremediation versus other processes (e.g., abiotic chemical precipitation) to the removal of metal(loid) s from the contaminated water, unvegetated controls were prepared. Here, a light brownish precipitate was observed within the MFS. For these non-vegetated controls, the difference between metal(loid) concentrations at T0 and T3 or after 90-days indicated the contribution to metal removal by other (abiotic) processes. Based on these results a mass balance was performed to assess the respective contribution of phytoremediation (% P), which included both phytoextraction and

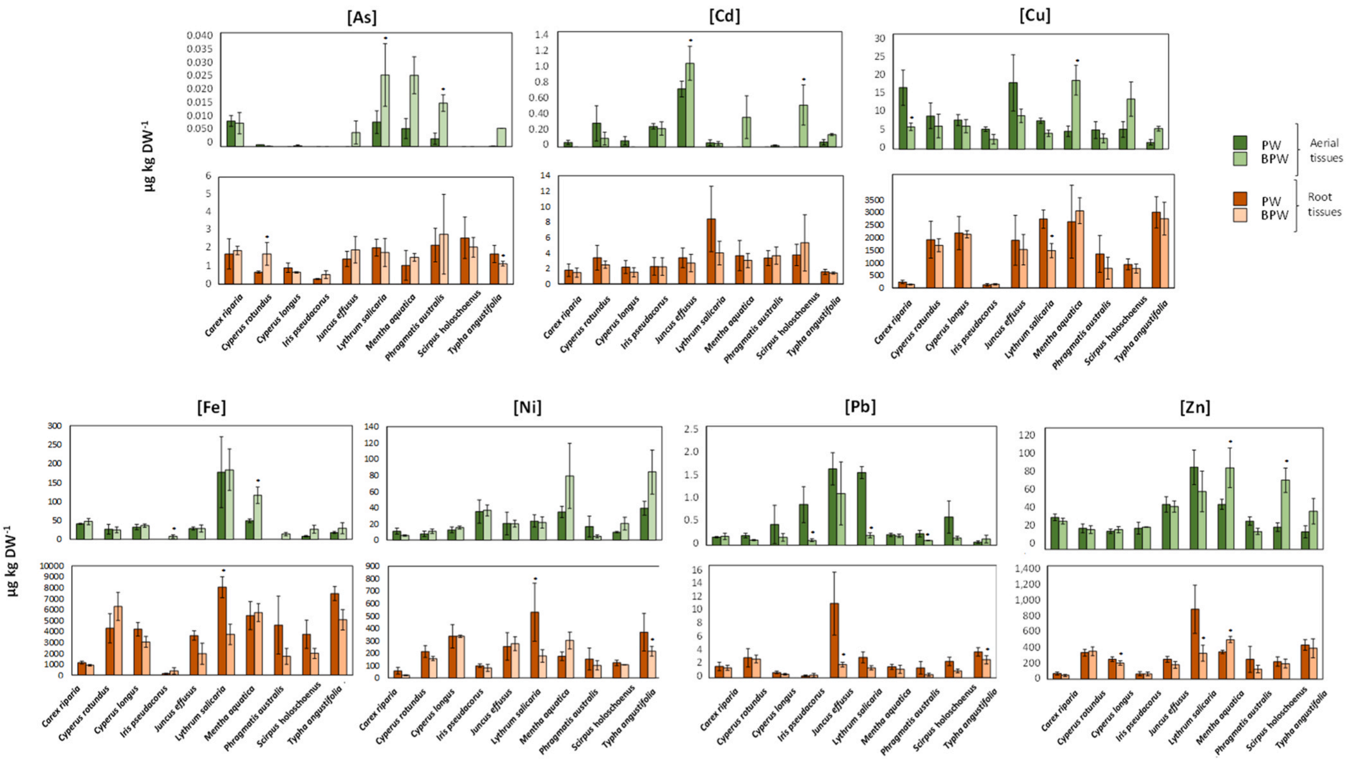

Fig. 2. Concentration of the target metal(loid)s in the two pool compartments (roots and aerial samples) as analysed by ICP-OES (Al, Cu, Pb, and Zn), and ICP-MS (As, Cd, and Ni). The samples displayed correspond to 90-days of exposure to PW (polluted groundwater) or BPW (bacteria + polluted groundwater) treatment. Data (vertical bars) represent the mean of three biological replicates (± standard deviation, SD). Results are expressed in µg Kg^{-1}DW. Asterisks indicate significant differences between the 2 treatments (PW and BPW) for each metal(loid) and plant analysed ($P < 0.05$, T-student test.

rhizostabilization, and other events (% O), to metal(loid)s removal (Supplem. Table S4). For As, Cd, Cu, Ni and Pb, removal was caused mainly by phytoremediation (>60%) for most plant species. For Pb, removal by phytoremediation accounted for more than 80% for all plants considered. In contrast, for Fe and Zn, removal due to other events was predominant for all plant species (<20% phytoremediation), except for *C. rotundus*. A similar phenomenon was observed for Cd in *T. angustifolia* (28% phytoremediation).

Bioconcentration and transference factors

Tables 2 and 3 show the bioconcentration (BCF) and transference (TF) factors calculated for the 10-aquatic species after 90 days of exposure to the real polluted groundwater (PW treatment). Results for the BCF values were very variable depending on the element and plant considered (Table 2). Per element and species, the three macrophytes with the greatest BCF values are highlighted in bold and numbered. The highest BCF values for Cu, Fe and Zn were reported for *L. salicaria*, *T. angustifolia,* and *M. aquatica*. *L. salicaria* was also among the first three positions with the highest BCF for all the studied elements: *M. aquatica* also for Cd, and *T. angustifolia* also for Ni and Zn. Other plants with relatively high BCF included *J. effusus* for Pb and *P. australis* and *S. holoschoenus* for As. Regarding the TF values, they were below the unit for most species and elements analysed (Table 3). Interestingly, *I. pseudacorus* showed, for all elements, higher TF values than the rest of the species, particularly for Pb with a TF ~3, denoting hyper-accumulation capacity for this element in the aerial compartment. *C. longus* and *L. salicaria* also exhibited a higher TF value for Pb than other plant species. *C. riparia* generally presented higher values of TF than the other plants, especially for Zn.

Discussion

In the present study the metal(loid) attenuation ability of 10 emergent macrophyte species, native of the European central and southern regions, was investigated using real groundwater from an industrial site over a 90-day exposure period. Previously reported work was generally performed either with one or few aquatic species [20], for rather short

Table 2
Results for the calculated BCF for the metal(loid)s under study in the ten aquatic species exposed to the PW treatment, after 90 days in the greenhouse.

Metal(liod)s	Plant used in the study									
	C. riparia	*C. rotundus*	*C. longus*	*I. pseudacorus*	*J. effusus*	*L. salicaria*	*M. aquatica*	*P. australis*	*S. holoschoenus*	*T. angustifolia*
As	74.24	29.37	40.29	12.47	61.33	**89.04**[3]	46.07	**95.69**[2]	**112.90**[1]	73.90
Cd	12.64	24.45	15.63	16.27	24.26	**60.33**[1]	**26.22**[3]	23.91	**26.82**[2]	11.06
Cu	4.42	34.94	39.80	2.43	34.44	**49.92**[2]	**47.97**[3]	24.66	17.13	**54.79**[1]
Fe	108.58	413.11	399.79	11.54	327.16	**780.27**[1]	**538.97**[3]	424.31	342.78	**712.43**[2]
Ni	7.95	29.08	**46.10**[3]	13.60	34.72	**72.51**[1]	23.89	20.90	16.61	**50.49**[2]
Pb	82.98	147.75	38.89	13.80	**551.83**[1]	**150.95**[3]	80.57	73.36	120.55	**192.57**[2]
Zn	3.95	18.91	14.24	3.78	14.12	**50.09**[1]	**19.38**[3]	14.23	12.51	**24.45**[2]

In bold, for each element, the plant species with the highest values for BCF (per row, three species per contaminant are highlighted).[1, 2, 3]Indicate the three highest BCF values in decreasing order for each element.

Table 3
Results for the calculated TF for the metal(loid)s under study in the ten aquatic species exposed to the PW treatment, after 90 days in the greenhouse.

Metal(liod)s	Plant used in the study									
	C. riparia	*C. rotundus*	*C. longus*	*I. pseudacorus*	*J. effusus*	*L. salicaria*	*M. aquatica*	*P. australis*	*S. holoschoenus*	*T. angustifolia*
As	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
Cd	0.03	0.08	0.03	**0.11**	**0.21**	0.01	0.00	0.00	0.00	0.04
Cu	0.07	0.00	0.00	0.04	0.01	0.00	0.00	0.00	0.01	0.00
Fe	0.02	0.00	0.01	0.04	0.01	0.01	0.01	0.00	0.00	0.00
Ni	**0.19**	0.04	0.04	**0.35**	0.08	0.04	**0.20**	0.11	0.08	0.11
Pb	**0.11**	0.07	**0.59**	**3.25**	**0.15**	**0.53**	0.02	**0.18**	**0.26**	0.02
Zn	**0.46**	0.06	0.07	**0.32**	**0.18**	0.09	**0.13**	0.11	0.10	0.04

In bold, the metallic elements and plant species displaying the highest values for TF.

incubation times [4], or for just a few elements [21]. In addition, most previous studies have used synthetic media instead of real contaminated water [13,14]. A better understanding of macrophyte tolerance under longer exposure times and using real environmental matrices is crucial for assessing the potential of these plants in phytoremediation and phytoattenuation strategies for aquatic systems.

The effectiveness of phytoremediation largely depends on the selection of the appropriate plants. These should be well-adapted to the local climate, actively take up or significantly remove or precipitate in the rhizosphere, one or more contaminants from the target matrix, and show normal growth and reproduction, that is, no signs of ecotoxicological effects [22]. Depending on the nature of the contaminant, these plants should have the capability to resist, degrade and/or adsorb pollutants [23,24]. Ideally, plants used for phytoremediation purposes grow rapidly, produce high biomass and possess an extensive root system.

After 90 days, plants exposed to the polluted water (PW, BPW) generally showed similar or reduced fresh aerial biomass compared to the controls (CW, CB) (Fig. 1). Milić et al. [25] suspected that the photosynthesis of *Davidia involucrata* was inhibited as the concentration of heavy metals in the substrate increased; in that study, concentrations of almost all metals were significantly higher in underground organs than in any other plant part. This mechanism could partially justify the decrease in biomass of the vegetative tissues after exposure to the contaminated water. Photosynthesis is inhibited due to metal-driven stress. The reaction mechanism is initiated in the rhizosphere; the plant triggers signals which induce remobilization of nutrients that result in wider root systems in detriment of reduced vegetative aerial parts. Induced senescence is behind this protective mechanism for multiple abiotic and biotic types of stress, and ultimately, activates a network of biochemical and molecular responses to rapidly adapt and tolerate the stress [26]. This could represent a frequent strategy in various wetland plants, including most of the macrophytes in this study (Fig. 2). Metal(loid)s are accumulated and immobilized in the root tissues to minimize distribution to the aboveground parts, particularly to the photosynthetic tissues, and, thus, avoid their damage [27]. Interestingly, aerial biomass of C. *riparia* and *I. pseudacorus* for all treatments was generally greater than for all other plants. These species were also characterized by lower metal(loid) accumulation in the roots compared to the majority of the macrophytes studied, more distinctive of an excluding tolerance strategy.

Although for most of the studied species the aerial biomass decreased when plants were exposed to polluted water, an opposite trend was observed for *C. riparia* (Fig. 1). For instance, fresh aerial biomass increased in *C. riparia* exposed to polluted water (PW) after 90 days. Ladislas et al. [21] tested *C. riparia* within their study and reported that biomass was not affected by metallic exposure. After screening 34 macrophyte species, Schück and Greger [4] concluded that *C. riparia* was one of the best performing species for removal of 4 metals and the only one characterized by greater biomass after metal exposure. Aerial biomass for *C. riparia* was significantly larger for the bioaugmented treatments (CB and BPW) with respect to their non-bioaugmented counterparts (CW and PW), indicating a potential beneficial effect of the PGPR. For *M. aquatica* bioaugmentation improved aerial biomass (BPW) regarding the non-bioaugmented polluted treatment (PW). Several studies have suggested a positive effect on metal removal and biomass production when plants were bioaugmented with microorganisms as part of a "phytobial" treatment [28,29]. Tara et al. [30] bioaugmented *P. australis* and *Typha domingensis* in FTWs with a bacterial consortium consisting of three strains to treat industrial textile wastewater. The authors observed improved removal efficiency following bioaugmentation. Fahid et al. [31] observed for *Cyperus laevigatus* a 73.48% reduction in hydrocarbon concentrations after phytoremediation combined with bioaugmentation. In most cases, the plant biomass increased after bioaugmentation. Plant tolerance to stress can be improved by PGPR that promote both plant growth and development through the release of phytohormones. Nawaz et al. [14] found that *P. australis* in the presence of synergistic bioaugmented bacteria achieved higher root and shoot growth, as compared to plants without inoculation. It should be noted that the studies previously described report a positive effect on one type of plant and, thus, may not apply to other species. In the present study, a consistent general phytobial effect for all species was not observed. Yet, for *C. riparia* and *M. aquatica* bioaugmentation also improved growth and, by extent, tolerance of the plant to the metal(loid) contamination. These results suggest that the suitability of phytobial treatment needs to be addressed case by case at an early assessment stage, but also has potential to improve plant performance in the presence of mixed contaminations.

In addition to metal(loid) concentrations and typical parameters related to plant growth (such as, temperature and light), soil/water pH and salinity can significantly influence metal(loid)s uptake and plant growth, ultimately impacting the phytoremediation performance [10]. For PW and BPW treatments, where plants were exposed to polluted water, a gradual pH increase and a concomitant decrease in EC was observed as time progressed (Supplem. Figs. S2 and S3). The general pH and EC patterns reported for most of the macrophytes exposed to polluted water can be attributed to the enhanced uptake of nutrients by the plants, including the metal(loid)s, as well as to the biological and physicochemical binding of pollutants to the roots and soil particles [14, 17]. Strikingly, for control treatments of *I. pseudacorus* (CB and CW), an initial acidification at T1 (15 days) and a later recovery at T3 (45 days) was observed. The initial pH decrease could be associated with the release of some acidic compounds in the rhizosphere by *I. pseudacorus* as the plant becomes exposed to the new medium. Once the influence of these compounds ceased, the pH of the water returned to its original value. Various macrophytes have been described to release bioactive compounds from the roots that may either enhance solubility, sorption and/or sedimentation processes in FTW. For instance, several studies reported the positive effect of citric acid, favouring the metals complexation and reducing their free mobility in plants, positively impacting the biomass production [13,32].

In addition to the root/shoot compartmentalization, metal(loid) concentrations were measured in the polluted water to assess phytoremediation/-attenuation performance. After 45-days, there was practically complete removal of all elements from the polluted water. For most elements, complete removal was achieved already after 15 or 30 days. According to the mass balance conducted, the contribution of phytoremediation mechanisms (such as enhanced precipitation, adsorption, or uptake by plants) accounted for more than 60% of As, Cd, Cu, Ni, and Pb removal, whilst abiotic mechanisms contributed to approximately 80% removal of Fe and Zn. It should be noted that a brownish precipitate was observed for most MFS. This form of precipitation has been previously described in similar studies and is attributed to the formation of iron (III) oxides/oxyhydroxides [33]. This agrees with the redox potential measured, which was indicative of oxidic conditions. Thus, it is probable that Zn removal was largely due to co-precipitation with the iron (III) oxides/oxyhydroxides. In their study with the floating water hyacinth (*Eichornia crassipes*), Palihakkara et al. [33] concluded that removal of Cu and Cd from water was mainly attributable to phytoremediation mechanisms and that, at neutral pH, no interactions occurred between Cd and Cu and the iron (III) oxide/oxyhydroxide precipitate observed. Similarly, an enhanced precipitation of Cd and Cu due to the iron precipitate was not observed in this study.

Metal(loid) uptake and compartmentalization by the macrophytes was assessed by means of BCF and TF. Concentrations of metal(loid)s in the roots were generally between 10–100 times larger than in the shoots. Thus, the species analysed in our work can be considered "underground accumulators"; this is in accordance with previous studies [14,25]. Sawidis et al. [34] stated that the increased accumulation of metal(loid)s in roots and rhizomes may be the result of the large intercellular air spaces that characterize their cortex parenchyma. The preferred metal (loid) accumulation in the roots supports the general notion that wetland plant species are very useful for phytostabilization rather than phytoextraction strategies [25]. Newete and Byrne [9] also reported that the extent of the root system affects the ability of macrophytes to remove metal pollutants, with fibrous root systems being superior to taproot systems due to their large surface area. This was the case for all ten macrophytes investigated. Generally, a BCF equal to greater than 10 is indicative of hyper-accumulative plants [35]. It should be noted that hyperaccumulators usually hyperaccumulate one or two elements and, thus, are not *per default* suitable to deal with contaminations by multiple elements. The BCF for all plants and elements analysed ranged generally between 10 - < 100, except for Fe, with values between 100–780 for the majority of species. Exceptionally, *J. effusus* showed a BCF of 552 for Pb. However, the species *L. salicaria*, *T. angustifolia* and *M. aquatica* presented the best BCF balance when all elements were considered.

The TF determines the ratio of metal(loid)s distribution between shoots and roots and, in turn, the potential use of a plant for phytoextraction ($\geq$1) or phytostabilization (<1) strategies [35]. In general, the TF was below 0.2 for most elements and plant species. Hence, the macrophytes studied are in general more appropriate for phytostabilization strategies. Wetland species tend to accumulate the bulk of trace elements in their underground organs, supporting rhizostabilization mechanisms [36]. An exception to this was *I. pseudacorus* with a TF for Pb of 3.25. Han et al. [37] studied the potential capacity of two Iris species, *I. lactea* and *I. tectorum*, in relation to Pb tolerance and phytoremediation mechanisms. Iris are widely distributed perennial species and are common ornamental plants; this is an additional valuable feature for phytorestoration strategies [37,38]. Other species with relatively large TF, yet below 1, included *C. longus* (0.59) and *L. salicaria* (0.53) for Pb, as well as *C. riparia* for Zn (0.46). For these species, harvesting the aerial part could be a suitable approach to further remove Pb and Zn from the target matrix, respectively.

Altogether, our results suggest that various of the species studied fulfil at least one of the criteria for phytoremediation and could be used to attenuate metal(loid) contaminations in aquatic systems or restoration of river margins. For instance, *C. riparia* and *I. pseudacorus* aerial biomass was not affected when exposed to the polluted water, whereas a significant decrease was observed for the other species. Hence, these plants are highly tolerant to mixed metal(loid)s contaminations and suit for the phytorestoration purposes. The potential of *C. riparia* for phytoremediation approaches was previously reported in other studies [4, 21]. The high TF of Pb for *I. pseudacorus* and metal(loid) tolerance, as shown by similar aerial biomass to controls, makes this plant also interesting for phytoextraction. In contrast, *L. salicaria, M. aquatica, S. holoschoenus,* and *T. angustifolia* showed relatively high BCF for various metal(loid)s, thus, having potential for phytostabilization strategies. *S. holoschoenus* also showed a good balance between metal(loid) tolerance and uptake, biomass stability with higher BCF for multiple metal (loid)s. The potential of this macrophyte for phytoremediation has been explored barely in the past [25]. It should be noted that for phytoextraction it can be more effective to harvest plants with greater above-ground biomass and moderate tissue concentrations of the pollutant of interest, rather than plants with lower biomass but higher tissue concentrations [39,40]. Thus, multiple phytostrategies may be possible for the same plant depending on the final aim of the project (e. g., restoration, stabilization, and extraction) [41].

Conclusion

The ability of the ten emergent macrophytes to tolerate and uptake metal(loid)s exposed to a real industrially polluted groundwater for 90-days differed significantly depending on the species considered. Six out of the ten candidates fulfilled at least one criterion for phytoremediation, including *C. riparia*, *I. pseudacorus*, *L. salicaria*, *M. aquatica*, *S. holoschoenus*, and *T. angustifolia*. In general, metal(loid)s accumulation occurred in the roots with little transfer to the shoots, suggesting that the investigated macrophytes are better suited for phytostabilization rather than phytoremediation strategies. Among all, *C. riparia* and *I. pseudacorus* showed higher tolerance to the mixed contamination than the rest of macrophytes as aerial biomass for these species was not affected by the exposure to the polluted water. These species tended to accumulate less metal(loid)s in the roots, being particularly promising for phytorestoration of river margins. The phytobial treatment did not produce a consistent general enhancement of metal(loid) uptake and growth but did show improved tolerance for *C. riparia* and *M. aquatica* when exposed to the polluted water. Thus, bioaugmentation approaches may improve plant performance in the presence of mixed contaminations, but this needs to be further assessed for each combination of PGPR, plant and contamination.

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Author statement

B. Velasco-Arroyo: original draft preparation, lab work and data analyses; **S. Curiel-Alegre** and **A.H.A. Khan**: Methodology, lab work, and data analyses; **C. Rumbo**: microbial analysis; **D. Pérez-Alonso**: lab work, and statistical revision; **C. Rad**, and **R. Barros**: conceptualization, text revision, and supervision. **H. de Wilde** and **A. Pérez-de-Mora**: site characterization and groundwater sampling, manuscript revision.

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Rad Carlos: Project administration, Resources, Software, Supervision, Writing – original draft, Writing – review & editing. **De Wilde Herwig:** Conceptualization, Data curation, Formal analysis, Methodology. **Rumbo Carlos:** Formal analysis, Investigation, Methodology. **Daniel Pérez-Alonso:** Investigation, Methodology, Resources. **Barros Rocío:** Investigation, Supervision, Funding acquisition, Writing –

original draft, Project administration, Writing – review & editing. **de-Mora Alfredo Pérez:** Data curation, Formal analysis, Methodology. **Velasco-Arroyo Blanca:** Data curation, Formal analysis, Writing – original draft, Conceptualization, Investigation, Project administration, Validation, Visualization. **Curiel-Alegre Sandra:** Data curation, Formal analysis, Investigation, Methodology, Resources, Software. **Khan Aqib Hassan Ali:** Investigation, Software, Writing – original draft, Writing – review & editing, Formal analysis, Visualization.

Declaration of Competing Interest

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2023.12.003.

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