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Optimizing the growth conditions of the fern *Pteris vittata* maximizes its ability to phytoextract arsenic from drinking water in multiple cycles

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ABSTRACT

The presence of arsenic (As) in drinking water poses a significant threat to public health and results in increased costs of water purification. Water phytofiltration using the As hyperaccumulator fern *Pteris vittata* is a promising green technology. However, for large-scale application, a fast uptake of As and the reuse of ferns in multiple cycles is necessary for an efficient As removal from drinking water at sustainable cost. This study investigated the efficacy of *P. vittata* in the treatment of spring water contaminated with about 60 $\mu g\ L^{-1}$ of geogenic As which is usually treated using costly chemical-physical filtration systems. To enhance the efficiency and reproducibility of *P. vittata*-mediated As phytofiltration, different key parameters were optimized including: i) ferns age, ii) phosphate (Pi) supply during ferns propagation, iii) As removal during subsequent phytofiltration cycles. Thus, As concentration in water and ferns grown individually was monitored over time. Our results indicate that As removal using old ferns was faster than using young ferns. Pre-treating the ferns with a phosphorus (P)-free solution enhanced As uptake and using a P-free solution in conjunction with old ferns allowed 98 % of As removal within 1 day. Moreover, As uptake further increased in subsequent phytofiltration cycles, and this efficiency was maintained when growing multiple ferns in the same tank. Altogether, these results definitively show that this strategy is an effective solution for treating spring water contaminated with geogenic As and opens the way for the scalability of this plant-based As phytofiltration technology.

1. Introduction

Arsenic (As) contamination is a worldwide problem due to its geogenic and anthropogenic sources, affecting both soil and water

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(World Health Organization 2018a, 2018b). According to World Health Organization (WHO) advice, European directories indicate that As concentration in drinking water must be below 10 μg L^{-1} (Council Directive 98/83/EC). Thus, several efforts have been made to improve the sustainability of As filtration from water using improved chemical physical technologies, such as adsorption coagulation and membrane. However, these technologies require expensive management, limiting the availability of uncontaminated water.

In this context, developing Nature-based Solutions (NbS) that can efficiently remove As from water could help managing water contamination and reduce treatment costs. *Pteris vittata* L. (*P. vittata*), commonly known as Chinese brake fern, is a well-established As hyperaccumulator plant species able to accumulate up to 27,000 mg kg^{-1} As in its fronds (Ma et al., 2001; Wang et al., 2002). *P. vittata* grows in a wide range of soil types under both subtropical and temperate conditions, as well as in hydroponic culture, showing high tolerance to As levels that are toxic to most plants (Natarajan et al., 2008; Antenozio et al., 2022). These features contribute to its effectiveness in removing As from contaminated water and soil, highlighting its potential as a model species for As phytoremediation strategies. In detail, *P. vittata* roots can uptake arsenate (AsO_4^{3-}; AsV) and arsenite (AsO_3^{3-}; AsIII), which are then translocated to the fronds and sequestered in vacuoles (Yamazaki et al., 2008). A few authors have investigated the phytofiltration efficiency of *P. vittata* for As in hydroponic cultures, showing promising results and providing insights into the mechanisms underlying As uptake. Due to its chemical analogy with phosphate (PO_4^{3-}, Pi), an essential nutrient element for plants, As in the form of AsV is absorbed through the Pi transporters (Di Tusa et al., 2016; Cao et al., 2018; Sun et al., 2020). Otherwise, As can be absorbed in the form of AsIII through the aquaporins, small channels that allow passive uptake of water into the roots (He et al., 2016). Both AsV and AsIII are the most common

Table 1
Literature overview of As phytofiltration in hydroponic *Pteris vittata* trials.

Scientific contributions	Initial As concentration	Optimal Phosphate [Pi] conditions tested	Fern Age	Time (days) to reduce As below 10 μg/L	Water volume (L) per plant	Plant density (n. of plants/ total volume L)	Multiple cycles with the same ferns	Arsenic removal rate (μg/d)
Tu et al. (2004)	46 μg/L nutrient solution added with As, N, P	P-free (HN−P) solution	3-months-old (young) and 12 months old (old)	3	0.6	1–3/0.6	2 cycles	7
Huang et al. (2004)	200 μg/L nutrient solution added with $CaCl_2$, P,^{73}As	0 μM	fern seedlings 10 cm high and 30–50 mL of root volume	1	0.8	1/0.6	3 cycles	158
Elless et al. (2005)	14 μg/L groundwater contaminated by As from pesticides	Not considered	ferns 10 cm tall with 5–6 fronds	84	5.5	8/45	Not considered	8572
Natarajan et al. (2008)	130 μg/L As contaminated groundwater added with 0.25 strength Hoagland's solution	P = 15 % of 0.25 strength Hoagland solution	3-months-old (young)	28	30	1–4/30	4 cycles	180
Santos et al. (2008)	145 μg/L nutrient solution added with As and/or P	134 mM Pi during acclimation + 66 mM Pi after As exposure	45-, 90- and 180-days-old plants	35	8	1	Not considered	30
Natarajan et al. (2009)	130 μg/L groundwater contaminated by As from pesticides added with N and P	12.2 μM (Low Pi)	6–7-months-old	35	15	1/15	Three cycles	900 (II cycle) and 225 (III cycle)
Santos et al. (2010)	126 μg/L As contaminated from pesticides water added with P	0 μM	45-days-old plants	32–74	18	1	Not considered	55
Natarajan et al. (2011)	140 μg/L groundwater contaminated by As from pesticides added with 0.25 strength Hoagland's solution	12.2 μM (Low Pi)	Young ferns 6–8 fronds stage	42	18	32/600	Two cycles	1857
Huang et al. (2015)	50, 500, 1000 μg/L added to solution	Not considered (low nutrient content)	2–4-month-old ferns	1–5	4	4/15 and 16/15	Not considered	150
Marzi et al. (2021)	59.705 μg/L spring water naturally contaminated with geogenic As	Not considered	Young ferns 6–8 fronds stage	7	15	1	Two cycles	127.5

inorganic forms of As in water and their respective amounts vary depending on environmental conditions (Smedley and Kinniburgh, 2002; Bednar et al., 2004). Once inside the root, both AsV and AsIII are loaded into the xylem and translocated to the aerial organs (Su et al., 2008). Proofs for a competitive interaction for transport between AsV and Pi have been provided for *P. vittata* plants grown in hydroponic culture, as high Pi concentrations (500 μM) inhibit As uptake, while low Pi content (20 μM) results in increased As influx (Wang et al., 2002). Indeed, pre-treating ferns without or with low Pi concentrations (10 μM) in water improves phytofiltration performances in *P. vittata* (Yamazaki et al., 2008). However, in most studies, ferns were grown in water or nutrient solutions artificially supplemented with varying concentrations of As (Tu et al., 2004; Huang et al., 2004, 2015), whereas only a few studies employed As-contaminated water collected directly from natural or anthropogenic sources (Natarajan et al., 2008, 2009, 2011; Santos et al., 2010; Marzi et al., 2021).

Depending on fern size and age, the time required for 99 % removal of As from nutrient solution supplemented with As, may span from about 35 days to 1 day. In general, in terms of roots and fronds biomass, As removal efficiency significantly increases in ferns with an extended root system and well-developed fronds (Natarajan et al., 2008, 2009, 2011; Marzi et al., 2021). Despite this, the time required for the removal of As concentrations in drinking water below the legal limit for (10 $\mu g\, L^{-1}$) ranges from about 84 days (Natarajan et al., 2008, 2009, 2011; Santos et al., 2010) to 7 days (Marzi et al., 2021) and remains too long to meet the standards for practical application (Table 1). So far, only a few studies have explored the reuse of ferns across consecutive As phytofiltration cycles. Interesting results have shown that by reusing the same ferns for up to three cycles of As phytofiltration, As removal increases according to the increase of ferns size (Natarajan et al., 2008, 2009, 2011; Marzi et al., 2021). All these findings make the use of *P. vittata* a promising tool for the development of green technologies to remove As from water. However, further studies are needed to assess whether As removal efficiency is maintained over time in subsequent cycles and whether this efficiency is also maintained when growing multiple ferns in the same tank, which may undergo nutrient competition limiting the efficiency of As removal (Tu et al., 2004; Natarajan et al., 2008, 2011; Huang et al., 2015). Addressing these aspects could provide hallmarks for the identification of the ideal conditions for efficient As phytofiltration, that may support the scale-up and the application of this approach (Elless et al., 2005). Furthermore, the reuse and valorization of ferns waste biomass have been successfully investigated (Eze and Harvey, 2018; Bavasso et al., 2023; Mazzeo et al., 2023), promoting the sustainable application of this NbS.

In the present study, we maximized As uptake by *P. vittata* grown on spring water contaminated with geogenic As, in order to evaluate its potential application in large-scale remediation strategies. We used ferns to treat spring water contaminated with geogenic As focusing on four main aspects: i) the optimal size and age of the ferns for a fast As removal; ii) the effect of pre-treatment with low phosphate (Pi); iii) the efficiency of consecutive phytofiltration cycles by reusing the same ferns; iv) the use of multiple *P. vittata* ferns in the same tank. Although our results provide useful information for the large-scale application of this phytoremediation strategy, the critical aspect remains the sustainable management and disposal of *P. vittata* biomass. Our results confirm that the roots primarily act as a transient organ for As uptake and transport, rather than as a long-term storage site (Antenozio et al., 2022). Consequently, root biomass has low As content and can be safely used in secondary applications, such as composite reinforcement materials production and as adsorbent for organic compounds (Mazzeo et al., 2022, 2023; Bavasso et al., 2023). By contrast, fronds represent the main site of As accumulation, and ensuring their safe disposal or valorization remains challenging (Eze and Harvey, 2018). Further research is required to identify efficient and cost-effective strategies for As extraction and recovery from fronds, thereby enabling their reuse and reducing the environmental risks associated with biomass disposal.

2. Materials and methods

2.1. Growth of Pteris vittata plants under hydroponic conditions

The propagation and growth of *Pteris vittata* L. plants was performed in the greenhouse under controlled conditions, as previously described (Marzi et al., 2021; Antenozio et al., 2021, 2022). Sporophytes with 4–6 fully developed fronds and expanded roots were moved to the hydroponic system. Tap water (Table S1; https://www.gruppoacea.it/al-servizio-delle-persone/acqua/acea-ato-2/la-qualita-della-tua-acqua), added with fertilizers (Hydrogrow X + Y, Cellmax) was used for ferns growth. EC monitoring during *P. vittata* growth was performed with a portable Conductivity Meter (Milwaukee EC60-Milwaukee Instruments, Inc, Szeged, Hungary) and fertilizers (Hydrogrow X + Y, Cellmax, https://www.cellmax.eu/en/products/hydro-grow-xy-set) were added when required. Water electroconductivity (EC) was set to 2 (mS/cm), while pH was naturally established at 8.4 by ferns themselves. In these conditions, ferns needed about 2–3 months and 4–6 months to reach "young" and "old" growth stages, respectively. Young ferns were characterized by 20–25 root length and 6–8 frond stage, while old ferns by 30–35 cm root length and 10–12 frond stage. These age ranges were chosen based on the specific growth cycle of *P. vittata* under our controlled conditions. Plants at 2–3 months show limited frond development and root establishment, whereas those at 4–6 months display larger frond biomass and a fully developed root system. This classification allowed us to set up non-invasive parameters for plant selection and reproducibility. In this framework, the age and biomass of ferns are strictly correlated.

For plant growth and for all the phytofiltration experiments, a Deep Water Culture (DWC) hydroponic system was used, consisting of 4 or 13 L tanks equipped with an air pump to oxygenate water used for the growth of single ferns. When using multiple ferns, the tank contained 39 L of water and hosted 3 ferns.

During the propagation/growing phase ferns were grown in tap water supplied with fertilizers; thus, before the phytofiltration cycle, ferns with similar characteristics were selected, roots were washed with water and then were placed in experimental tanks. For all phytofiltration experiments, no treatment to adjust the pH conditions was applied, nor were fertilizer added to the naturally As contaminated spring water during the experiments.

2.2. Phytofiltration experimental set up

2.2.1. Water and plant material

All the experimental trials were performed by using *P. vittata* plants provided and propagated as previously described by Marzi et al. (2021). According to Marzi et al. (2021), for the phytofiltration trials naturally As-contaminated spring water was collected from Varano waterwell, located in Nepi (Viterbo, Lazio, Italy, 42°15'29.2" N 1218'24.2" E). This waterwell is used to draw drinkable water which has As contamination above legal limits (10 $\mu g\,L^{-1}$), hence needs to be treated before use.

2.2.2. Phytofiltration cycles with young and old ferns

Five young ferns and five old ferns were selected and individually transferred into tanks filled with 13 L of naturally As contaminated spring water. Five additional tanks containing the same volume of naturally As contaminated spring water, without ferns, were used as controls. To evaluate the As removal and accumulation in plants over time, As content was measured by inductively coupled plasma mass spectrometry (ICP-MS) in water at 0, 1, 2, 7, 15, 30, 60 days, and in fronds and roots separately collected at 0 and 60 days.

2.2.3. Phytofiltration cycles with young ferns pre-treated with different phosphate (Pi) conditions

Young ferns were selected and pre-treated by growing them for 15 days in individual tanks (n = 6 for each condition) filled with 4 L tap water added with 0, 10, 100, 1000 μM $NH_4H_2PO_4$, respectively. During the pre-treatment no fertilizers were used. Then each pre-treated fern was transferred individually into tanks filled with 4 L of naturally As contaminated spring water. Arsenic content was measured by ICP-MS in water at 0, 1, 2, 7, 15, 30, 60 days, and in fronds and roots separately collected at 0 and 60 days, to evaluate the phytofiltration indexes.

2.2.4. Reuse of ferns for consecutive phytofiltration cycles

Individual old ferns (n = 3) pre-treated for 15 days using tap water were transferred into individual tanks filled with 13 L of naturally As contaminated spring water. Arsenic content was measured by ICP-MS in water at 0, 1, 2, 3, 4, 7, 15 days, and in fronds at the beginning and at the end of each phytofiltration cycle.

After the first phytofiltration cycle, the same ferns were reused for 5 consecutive phytofiltration cycles, changing each time the treated with new untreated naturally As contaminated spring water. Arsenic content was measured by ICP-MS in water at 0, 8, 1, 2, 3, 7 days, as well as in fronds at the beginning and at the end of each phytofiltration cycle.

2.2.5. Phytofiltration cycles with multiple ferns in the same tank

The old ferns were pre-treated by growing them on tap water for 15 days without any fertilizer. Then they were transferred into two tanks (59x39x36.5 cm) containing 39 L of naturally As contaminated spring water, using 3 ferns in each tank. Three subsequent phytofiltration cycles were performed reusing the same ferns and changing each time the treated solution with new untreated naturally As contaminated spring water. Arsenic content was measured by ICP-MS in water at 0, 8, 1, 2, 3, 7 days, as well as in fronds at the beginning and at the end of each phytofiltration cycle.

2.3. Phytofiltration index calculation

Phytofiltration indices were calculated according to literature (Kumar et al., 2022; Nassazzi et al., 2023).

$$\text{Translocation factor(TF)} = \frac{[C](\text{shoot})}{[C](\text{root})} \tag{1}$$

where C(shoot) indicates the metal concentration (mg kg^{-1} DW) accumulated in the shoot part and C(root) indicates the metal concentration (mg kg^{-1} DW) accumulated in the root part.

Role: the TF describes the efficiency of As transfer from roots to shoots.

$$\text{Bioconcentration factor(BCF)} = \frac{[C](\text{plant tissue or aerial part})}{[C](\text{substrate})} \tag{2}$$

where C(plant tissue) indicates the metal concentration (mg·kg^{-1} DW) accumulated in the plant tissue (shoot or root or leaf) and C (substrate) indicates the metal concentrations (mg·L^{-1}) in the medium.

Role: the BCF indicates the plant's ability to absorb and accumulate As from the medium through its roots or aerial parts.

$$\text{Removal efficiency(RE\%)} = \frac{[Co] - [Cf]}{[Co]} x100 \tag{3}$$

where [Co] is the initial metal concentration and [Cf] is the final metal concentration in the soil (or water) after plantation.

Role: The RE% describes the percentage of As removed from the growing medium.

$$\text{Plant total burden (PTB)} = \sum_{i=1}^{n} Mi \quad x \quad Ci \tag{4}$$

where [Mi] is the dry biomass of plant tissues (roots, stem, leaves) and [Ci] is the metal concentration in the part "*i*".

Role: The PTB provides the total amount of As sequestered by the entire plant, taking into account both biomass and concentration.

2.4. Chemical analysis of arsenic content in Pteris vittata tissues

Fern tissues sampling was performed as follows: 1) the roots were washed with distilled water and about 3–4 g fresh tissues were collected for each fern; 2) fronds were collected and processed using the "pinna powder" method at the beginning and at the end of the phytofiltration cycle. The "pinna powder" method consists in the sampling of 1 pinna per frond per fern, in order to obtain a representative and effective quantification of As accumulation in the whole plant (Capobianco et al., 2022; Antenozio et al., 2024). Samples were completely dried in an oven set at 50°C (3 days). Subsequently, 100 mg (dry weight) of each sample was subjected to microwave-assisted acid digestion (Ethos Touch Control system with Q20 rotor; Milestone, Italy) at 180 °C for 30 min, using a 2:1 (v/v) mixture of HNO_3/H_2O_2 (nitric acid 65 %, Carlo Erba, Italy; hydrogen peroxide Suprapur, Merck, USA). The digested solution was then diluted 1:1000 with deionized water and filtered through 0.45 μm cellulose nitrate syringe filters (GVS Filter Technology, England).

The total concentration of As and other 30 macro-, micro-, and trace elements (the findings of which will be presented in a forthcoming paper) were determined using an inductively coupled plasma mass spectrometer (ICP-MS; PlasmaQuant MS Q, Analytik Jena, Germany) equipped with a glass nebulizer (0.4 mL min^{-1}; Analytik Jena, Germany). Calibration curves were generated by serially diluting 1000 ± 2 mg L^{-1} multi-standard stock solutions (Merck, USA). Two internal standards, yttrium and rhodium, were used to control nebulization efficiency. The limit of detection (LOD) for each element was determined as the mean plus three times the standard deviation of ten blank determinations. The As concentration (μg g^{-1}) in *P. vittata* tissues was calculated by dividing the As content by the dry weight of each sample.

2.5. Chemical analysis of total arsenic in water samples

Water samples (50 mL) were collected before and during phytofiltration cycles at different times. After filtration through a 0.45 μm PVDF, each sample was acidified to 1 % with extra-pure fuming nitric acid (Merck, Darmstadt, GE) and analyzed with a Perkin-Elmer NexION 300D ICP-MS spectrometer (PerkinElmer Inc., Shelton, CT, USA) operating with an ESI autosampler (PerkinElmer Inc., Waltham, MA, USA) and a U6000AT+ Teledyne Cetac Technologies ultrasonic nebulizer (Teledyne Cetac, Omha, NE, USA). The spectrometer working conditions (i.e., argon flowrates into the nebulization chamber and torch as well as plasma generator, quadrupole and detector potentials) were optimized on a daily basis using a setup solution containing Be, Ce, Fe, In, Li, Mg, Pb and U at a concentration of 1 μg L^{-1} for each element in HNO_3 1 %. Quantitative determinations of As were performed under standard mode and pulse detection conditions without activating the collision/reaction cell as the polyatomic interference 40Ar35Cl+ was not detected at the low chloride concentrations present in the examined samples. Internal calibration method was applied for quantitative determination using a blank solution containing HNO_3 1 %, five external standard solutions in the range of 1–50 μg L^{-1} and 20 μg L^{-1} of 115In as internal standard, the latter added to every examined solution. All standard solutions were prepared by diluting the corresponding stock 1.00 g L^{-1} solution (AccuTrace Reference Standard) and acidified to 1 % with extra-pure fuming nitric acid.

2.6. Chemical speciation of arsenic in water samples

A water sample (50 mL) was collected in January 2024 at Varano well near to Nepi (Viterbo, Lazio, Italy) and stabilized on site by adding n-hexane 97 % (VWR Chemicals BDH, Radnor, PA, USA) to prevent the oxidation of AsIII by atmospheric oxygen. Its speciation analysis was carried out by ICP-MS coupled with ion chromatography. In particular, separation and determination of As species were performed using isocratic elution at 1 mL min^{-1} with nitric acid 4 mM from a Dionex IonPac AS10 analytical column 4 mm× 250 mm (Thermo Fisher Scientific Inc., Waltham, MA, US) installed into a Shimadzu LC-10 AD chromatographic system (Shimadzu Corporation, Kyoto, JP). A U6000AT+ Teledyne Cetac Technologies ultrasonic nebulization system (Teledyne Cetac, Omha, NE, USA) was used to introduce the column effluent into a Perkin-Elmer NexION 300D ICP mass spectrometer (PerkinElmer Inc., Shelton, CT, USA). Quantification of the analytes was performed by standard additions of AsIII and AsV to the matrix. Stock solutions of AsIII and AsV were prepared from As_2O_3 99.5 % (Alfa Aesar, Ward Hill, MA, USA) and As_2O_5 99.9 % (Alfa Aesar, Ward Hill, MA, USA), respectively, in MQ-grade water (18.2 MΩ•cm) produced by a Millipore RiOs™ Essential 5 (Merck, Darmstadt, GE). A HNO_3 1 % (Merck, Darmstadt, GE) solution driven by an external peristaltic pump at the flow rate of 1.0 mL min^{-1} was mixed to the column effluent before the nebulization system to even out plasma responses to different species. Chromera software (version 4.1.2.6410, Shelton, CT, USA) was used for data collection and chromatographic peaks integration. ICP-MS operating conditions were daily optimized as described previously.

2.7. Statistical analysis

Statistical analyses were performed using Microsoft Excel and GraphPad Prism8. Differences between two groups were assessed using the Student's *t*-test and asterisks indicate the significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). One-way and two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests were used to compare different groups and different variables together; different letters indicate significant differences between groups ($P < 0.05$).

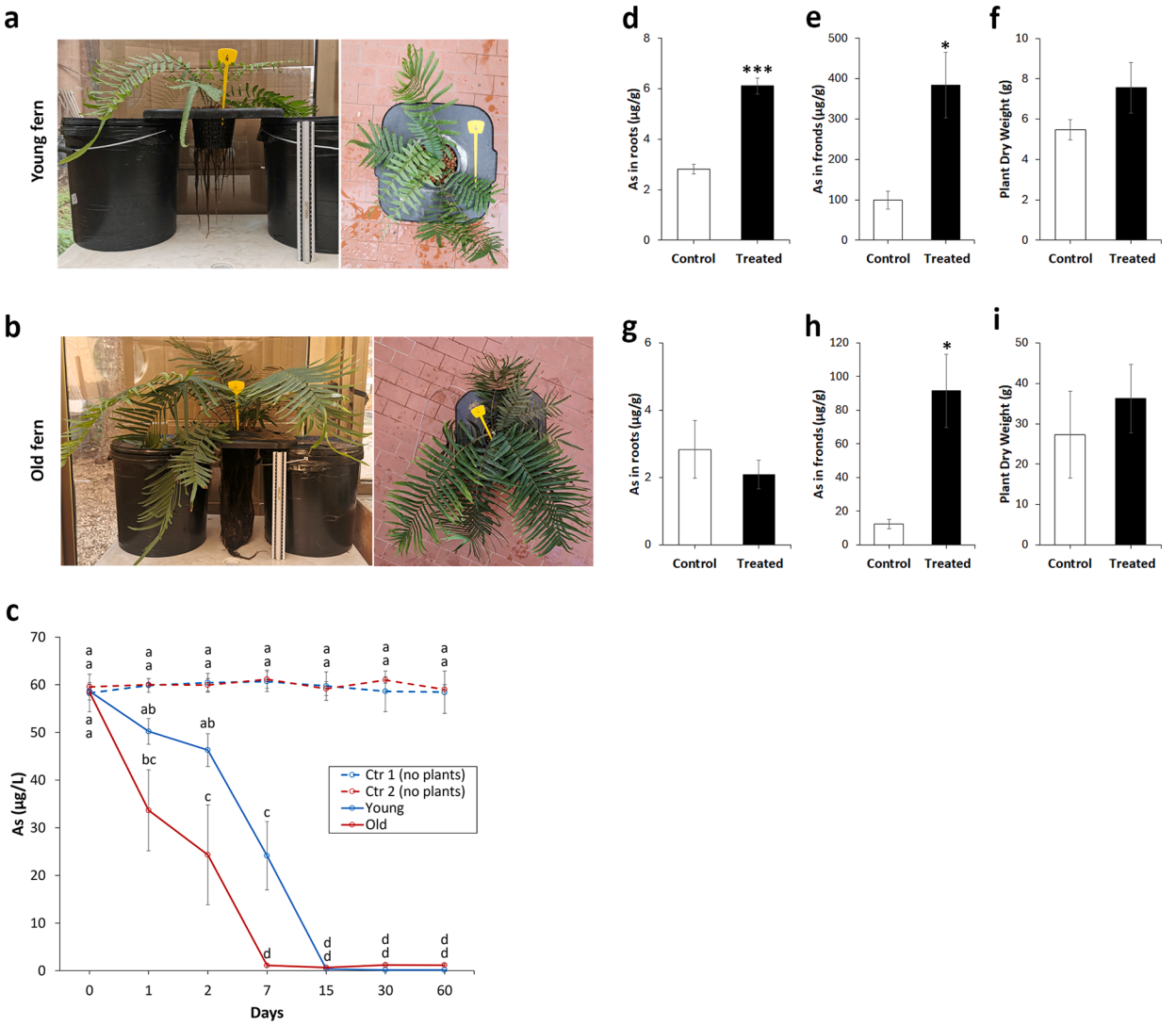

Fig. 1. Phytofiltration cycle with young and old ferns. Representative pictures of young (a) and old fern (b). Arsenic concentration at different timepoints in naturally As contaminated spring water treated with young and old ferns and in untreated controls (Ctr1 and Ctr2) (c). Analysis of the amount of As in the (d) roots and (e) fronds of young ferns at the end of the phytofiltration cycle. Total dry weight (gr) of young ferns at the end of the phytofiltration cycle (f). Analysis of the amount of As in the roots (g) and fronds (h) of old ferns at the end of the phytofiltration cycle. Total dry weight (gr) of old ferns at the end of the phytofiltration cycle (i). Data is presented as mean± SE (n = 5). In (c) significant differences between As concentration at different timepoints and between young and old ferns were calculated by two-way ANOVA followed by Tukey's test; different letters in the graph indicate significant differences ($P < 0.05$). Asterisks in the graphs (d-i) indicate significant differences as calculated using the Student's T-test (* $P < 0.05$; *** $P < 0.001$). Dashed blue line: Ctr 1 (no plants)- experimental control without young ferns; dashed red line: Ctr2 (no plants)- experimental control without old ferns; blue line: Young ferns; red line: Old ferns.

3. Results and discussion

3.1. Arsenic uptake from water increases with ferns size

The removal efficiency of As varies depending on factors such as the concentration of As in the water, the volume of water treated, and the size of the ferns. The main studies investigating the capacity of *P. vittata* to remove As from water are summarized in Table 1. In this study, we assessed the efficiency with which individual plants of different sizes and ages removed As over time in hydroponic culture, using 13 L of spring water naturally contaminated with 60 µg L^{-1} of geogenic As per plant (Marzi et al., 2021). We defined young and old ferns based on the number of fronds and root length (Fig. 1a, b); thus, we evaluated their As removal efficiency over time in hydroponic culture using ICP-MS analysis. By means of speciation analysis we also showed that the As in spring water was in the form of AsV, both organic and inorganic, while AsIII was not detected (Fig. S1). This is likely related to the chemistry of As which tends to be converted in AsV in water (Smedley and Kinniburgh, 2002; Bednar et al., 2004).

Young ferns reduced As concentration in water by 14 %, 21 %, 59 % and 99.3 % in 7 and 15 days, respectively, and it remained at 99.7 % of the initial concentration after 30 and 60 days (Fig. 1c). Old ferns showed a higher As removal efficiency compared to the young ones, as they removed 42 %, 58 % and 98 % of As in 2 and 7 days, respectively (Fig. 1c). After 1 day of phytofiltration, old ferns significantly decreased As content in water compared to the initial concentration (T0), while young ferns only decreased slightly the amount of As in water. After 2 days of phytofiltration, As removal was significantly higher in old ferns compared to the young ones, and this difference was maintained until 99 % of As was removed from water at 15 days of treatment. As expected, As concentration remained unchanged in the control tank without ferns (Fig. 1c). These results are comparable to those obtained by different authors (Table 1), that obtained the 98 % removal of As in less than 7 days using old ferns (Natarajan et al., 2008, 2009, 2011; Santos et al., 2010; Marzi et al., 2021).

The ICP-MS analysis of ferns tissues revealed that, after 60 days of phytofiltration, both young and old ferns accumulated up to 1007.705 ± 82.154 μg As plant^{-1} and 1098.004 ± 69.224 μg As plant^{-1}, respectively, as calculated by PTB (Table 2). Interestingly, both young and old ferns grown on control solution (i.e., tap water) accumulated small amounts of As (Table 2), likely due to the presence of As in trace in the fertilizer used during the initial phases of ferns propagation and the high tendency of *P. vittata* to accumulate As. Taking into account this preliminary As accumulation in control ferns, our results showed that approximately all the As contained in the 13 L of contaminated spring water was absorbed and accumulated in ferns. The young ferns accumulated about 6.109 ± 0.717 μg g^{-1} of As (mean ± SE) in roots (Fig. 1d), whereas the fronds accumulated up to 383.7 ± 181.4 μg g^{-1} of As (mean ± SE) after 60 days growth in hydroponic culture (Fig. 1e). The old ferns accumulated 2.085 ± 0.428 μg g^{-1} of As (mean ± SE) in the roots (Figs. 1g) and 91.475 ± 21.8 μg g^{-1} of As (mean ± SE) in the fronds (Fig. 1h) after 60 days. The highest As concentration (μg g^{-1}) found in young ferns is due to their lower biomass, compared to the old ones (Figs. 1f, 1i). The bioconcentration factor (BCF) for roots and fronds in young ferns grown in water containing As was 0.104 and 6.548, respectively, while it was 0.036 and 1.564 for roots and leaves in old ferns, indicating the highest capacity of fronds to accumulate As compared to roots. The translocation factor (TF) was 62.8 and 43.9 for young and old ferns, respectively, as expected for hyperaccumulator plant species (Table 2), indicating that ferns of both ages have high efficiency in translocating As from the roots to the fronds. Altogether, these results underline the remarkable capacity of *P. vittata* to uptake and accumulate As. Furthermore, this data indicates that measuring As removal from water provides a good approximation of As accumulation in *P. vittata*, enabling the As content in the ferns to be estimated non-destructively. Altogether, our results are in line with the phytofiltration indices and As removal rate per day for young and old ferns (Table 1). Prior studies compared ferns of different age but similar size, revealing that young ferns are more efficient in As removal than the older ones (Tu et al., 2004). However, under our experimental conditions and according to literature (Natarajan et al., 2008), old ferns have a higher biomass compared to the young ones and As uptake is faster in the former than in the latter. Taken together, these data indicate that the efficiency of As removal increases with roots and frond biomass.

3.2. Phosphate deficiency increases arsenic removal efficiency

Another major concern in phytofiltration is the accumulation of nutrients, particularly phosphorus (P), which are used to optimize fern growth. It has been reported that the uptake and transport of Pi and AsV are competitive through the same plasma membrane system. On the other hand, it has been shown that Pi starvation and low concentration of Pi enhance initial As uptake, while adding up to 150 μM Pi may increase this process in long-term trials (Santos et al., 2010). Thus, we evaluated the impact of pre-treating young ferns with different concentrations of Pi, on subsequent As uptake. Phosphate was supplied through a pre-treatment phase rather than added directly to naturally As contaminated spring water, to avoid alterations in the characteristics of this drinking water. Table S2 shows the physicochemical properties of the Varano waterwell As-contaminated spring water, including its low Pi content. After a 15-day pre-treatment period, ferns were transferred to naturally As contaminated spring water for the phytofiltration trial during which the removal of As from the water was monitored over time. Ferns pre-treated with water (Pi starvation) or with water containing 10 μM Pi showed a significant decrease in As concentration after 2 and 7 days, with 98 % of As removed in about 15 days (Fig. 2). Conversely, pre-treatment with 100 μM Pi or 1000 μM Pi significantly increased the time required to remove 98 % of As, from 15 to 30 and 60 days, respectively (Fig. 2).

These results indicate that As phytofiltration efficiency increases when ferns are exposed to Pi starvation, whereas increasing the Pi concentration to 1000 μM in water during pre-treatment decreased the As removal efficiency of ferns by four times compared to ferns treated only with water. These data are consistent with the finding that the As in the spring water used in this study was in the form of AsV, and align with previous studies which indicated that *P. vittata* plants grown in low Pi solution showed an increased As uptake (Wang et al., 2002; Yamazaki et al., 2008; Santos et al., 2008, 2010).

3.3. Old ferns deprived of Pi can remove As in 24 h and reusing them in subsequent cycles enhances their As removal efficiency

In order to assess whether the concurrent utilization of both optimal growth conditions can further decrease the time of As uptake, individual old ferns that had been pre-treated under Pi deprivation were transferred to spring water contaminated with geogenic As. The level of As in water was monitored over time. As shown in Table 3, Pi starvation significantly enhanced As removal from water by old ferns, reducing the time required to remove 98 % of As from 7 days to 1 day. The concentration of As further decreased after 2 days, reaching the concentration of 0.3 μg L^{-1} , that was almost stable for the subsequent 15 days.

The extant literature on the reuse of ferns for subsequent cycles is extremely limited, with the majority of the research having been conducted only up to four cycles (Table 1). However, these studies suggest that reusing the same fern for subsequent phytofiltration cycles improves As removal from water (Tu et al., 2004; Huang et al., 2004; Natarajan et al., 2008, 2009, 2011; Marzi et al., 2021).

Table 2
P. vittata phytoremediation efficiency indexes of young and old ferns at the end of the phytofiltration cycle.

Plant Phytoremediation Efficiency Indices					
	Initial As concentration (μg/L) in water	**PTB (μg)**	**BCF frond**	**BCF root**	**TF**
Control Young Ferns	0.513 ± 0.0136	205.397 ± 11.333	1.689	0.048	35.135
Treated Young Ferns	58.600 ± 1.85	1007.705 ± 82.154	6.548	0.104	62.808
Control Old Ferns	0.513 ± 0.0136	196.374 ± 21.994	0.211	0.048	4.355
Treated Old Ferns	58.500 ± 0.76	1098.004 ± 69.224	1.564	0.036	43.869

TF, translocation factor; BCF_{frond}, bioconcentration factor in fronds; BCF_{root}, bioconcentration factor in root; PTB, plant total burden.

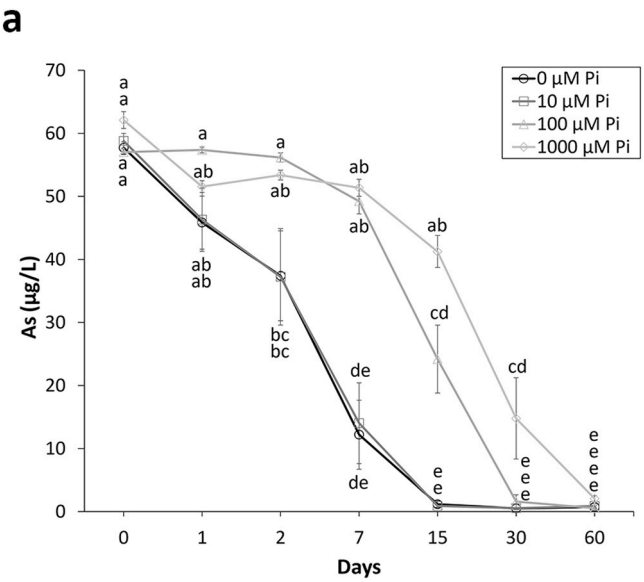

Fig. 2. Analysis of the amount of As in water over time during the phytofiltration cycle with young ferns pre-treated for 15 days with 0, 10, 100, 1000 μM Pi. Data is presented as mean ± SE (n = 6). Significant differences between As concentration at different timepoints and between ferns pre-treatments were calculated by two-way ANOVA followed by Tukey's test; different letters in the graph indicate significant differences ($P < 0.05$). Black line: 0 μM Pi; dark grey line: 10 μM Pi; grey line; 100 μM Pi; light grey line 1000 μM Pi.

To assess the possibility of reusing ferns for subsequent cycles, ferns that had been used in the first cycle were used in five subsequent phytofiltration cycles, reaching a total of six cycles. As shown in Table 3, for cycles 2, 3 and 4, after 8 h As removal reached 75 %, 80 % and 82 %, respectively. However, the As concentration remained slightly above the limit of 10 μg L^{-1} (Table 3). In the remaining two cycles, As removal significantly improved while As concentration fell below the legal limit (Table 3).

The increased efficiency of As removal from water was confirmed by the ICP-MS analysis of fronds, which showed an increased content of As at the end of each cycle (Fig. 3). Interestingly, As content significantly increased from cycle 0 (43.121 μg g^{-1}) -corresponding to T0 to the cycle 2 and 3 (201.701 μg g^{-1} and 265.244 μg g^{-1}, respectively), and further increased from cycle 4 (265.244 μg g^{-1}) to cycle 6 (317.967 μg g^{-1}). The BCF in fronds were 2.45, 3.34, 4.81, 4.30, 5.42, 5.51 as evaluated at the end of cycles 1, 2, 3, 4, 5, 6, respectively. At the end of the trial, PTB accounted for about 6000 μg As $plant^{-1}$ for each treated fern.

One of the most critical challenges in phytoremediation is minimizing the time required to effectively remediate a contaminated matrix. As demonstrated by the results presented in Table 2 and Fig. 3, under our experimental conditions As concentration was decreased below legal limits in less than 1 day in the phytofiltration cycles 1–4, while this process occurred in less than 8 h during cycles 5 and 6. These results definitely show *P. vittata* to have a consistent capacity to uptake As from water in subsequent cycles. (Natarajan et al., 2008). To date only one study, on water contaminated by As-based herbicide has demonstrated a comparable level of efficiency only in cycle 3. However, it took five weeks and two days respectively to go below 10 μg L^{-1}, in the first and second cycle (Natarajan et al., 2008).

3.4. Arsenic removal efficiency is maintained by increasing plant density

For an effective application of phytofiltration, multiple plants should be used at the same time. As it can influence the development and physiological function of plants via many pathways, planting density is a critical factor for phytoremediation. In soil high root density can improve ion uptake but can also increase competition between plants (Jacobs et al., 2018). Similarly, when several plants are grown together in hydroponic culture using the same solution, competition between the plants may limit the uptake of nutrients

Table 3
Analysis of As content in water over time during 6 subsequent phytofiltration cycles. Data are reported as mean ± SE (n = 3). Different letters indicate significant differences among sampling times at each cycle.

As in water [µg/L]								
	T0	**T8h**	**T1 d**	**T2 d**	**T3 d**	**T4 d**	**T7 d**	**T15 d**
C1	51.094 ± 0.094(a)		2.097 ± 2.446(b)	0.188 ± 0.091(b)	0.414 ± 0.033(b)	0.392 ± 0.002(b)	0.353 ± 0.039(b)	0.300 ± 0.000(b)
C2	60.300 ± 1.352(a)	14.700 ± 6.755(b)	0.866 ± 0.208 (c)	0.233 ± 0.057(c)	0.300 ± 0.000(c)		0.233 ± 0.057(c)	
C3	55.100 ± 0.888(a)	11.266 ± 6.028(b)	0.666 ± 0.251(c)	0.266 ± 0.057(c)	0.300 ± 0.000(c)		0.266 ± 0.057(c)	
C4	57.033 ± 1.040(a)	10.033 ± 8.707(b)	0.633 ± 0.230(c)	0.300 ± 0.000(c)	0.300 ± 0.000(c)		0.300 ± 0.000(c)	
C5	56.866 ± 2.478(a)	6.700 ± 5.243(b)	0.500 ± 0.100(bc)	0.300 ± 0.000(c)	0.300 ± 0.000(c)		0.266 ± 0.057(c)	
C6	57.000 ± 0.458(a)	5.966 ± 4.705(b)	0.566 ± 0.115(bc)	0.300 ± 0.000(c)	0.300 ± 0.000(c)		0.333 ± 0.057(c)	

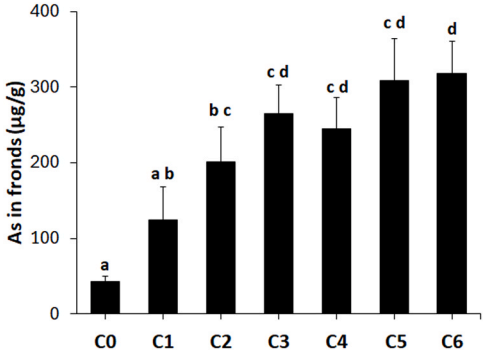

Fig. 3. Analysis of the amount of As in fronds over time during 6 subsequent phytofiltration cycles (C0-C6). Data is presented as mean ± SE (n = 3). Different letters indicate significant differences ($P < 0.05$) among different sampling timepoints at each phytofiltration cycle, respectively.

(Jia et al., 2020). Therefore, under these conditions, competition may reduce the As removal efficiency from water. In Tu et al. (2004), the authors showed that an increase of plant density did not further increase the As-depletion rate. To assess whether efficient removal of As was achievable when multiple plants were grown in the same tank, we kept the water to plant ratio constant. Three subsequent phytofiltration cycles were performed using 2 tanks each containing 39 L of As-naturally contaminated spring water and 3 ferns pre-treated under Pi deprivation for 15 days. As shown in Table 4, the amount of As removed significantly increased at 8 h, 1 and 2 days and was similar to the amount removed when ferns were grown individually. Accordingly, As removal increased in subsequent cycles and ferns absorbed up to 88 % of the As within 8 h in the cycle 3. These results were further confirmed by ICP-MS analysis, which showed that As concentration significantly increased in the fronds after each phytofiltration cycle (Fig. 4). Like phytofiltration cycles with single ferns, cycles using multiple ferns together allowed the removal of about 98 % of As from each tank in about 1 day, accumulating up to 209.987 ± 4.34 µg g^{-1} of As (mean±SE) in the fronds in the cycle 3. Accordingly, by incrementing the plant number but not volume of water to be treated, the efficiency of As removal increases (Natarajan et al., 2008). Altogether, these results showed that As removal efficiency is maintained when growing more ferns together, highlighting the efficiency, reproducibility and scalability of strategies based on *P. vittata* phytofiltration to remove As from water. Preliminary application has shown the applicability of this phytofiltration strategy (Elless et al., 2005).

These results show an improved As removal efficiency, supporting the notion that *P. vittata* is a good candidate for scale-up application of As phytofiltration, paving the way for more sustainable management of As contaminated water. The experimental design was normalized on a per-plant water volume basis to ensure comparability across treatments; however, defining an optimal planting density remains essential for large-scale applications. This parameter was not fully addressed here but it will be the focus of future studies aimed at scaling up this phytofiltration technology. Taken together, our results demonstrate that an increase in the above- and below-ground biomass of older ferns, whether taken individually or in groups, leads to faster As accumulation. Furthermore, this enhanced performance was sustained over multiple cycles. This efficiency may rely on the combination of different features, such as the increased radical biomass that expose augmented root surface to As contaminated water, the enlarged frond biomass that promotes a higher transpiration rate and develops vacuoles with increased number and dimension. Future studies will focus on these aspects and will shed light on the precise mechanisms involved in As uptake, translocation and storage, investigating the pivotal pathways regulating these processes and providing knowledge to further boost As phytofiltration using *P. vittata*.

4. Conclusion

Water contamination by As is a global issue. Chemical physical filtration devices are efficient in water depuration, but the costs for management and filters regeneration affect this service. This is especially true in emerging countries; thus, we need to find ways to remove As from water that are good for the environment. Our results demonstrate that phytofiltration using *P. vittata* is a biologically efficient and reproducible strategy for reducing As concentrations in naturally contaminated drinking water below the regulatory limit of 10 µg L^{-1} . The rapid phytofiltration of As by old ferns subjected to Pi deprivation, achieved within 1 day and maintained in

Table 4
Analysis of As amount in water over time during the 3 subsequent phytofiltration cycles using multiple ferns. Data are reported as mean ± SE (n = 2). Different letters indicate significant differences among different sampling times at each cycle, respectively.

As in water [µg/L]						
	T0	**T8h**	**T1 d**	**T2 d**	**T3 d**	**T7 d**
C1	54.800 ± 0.565(a)	18.200 ± 3.111(b)	1.150 ± 0.212(c)	0.250 ± 0.070(c)	0.200 ± 0.000(c)	0.200 ± 0.000(c)
C2	55.350 ± 1.060(a)	15.750 ± 6.858(b)	0.900 ± 0.424(c)	0.250 ± 0.070(c)	0.250 ± 0.070(c)	0.200 ± 0.000(c)
C3	56.000 ± 0.000(a)	6.750 ± 4.737(b)	0.500 ± 0.141(b)	0.250 ± 0.070(b)	0.300 ± 0.000(b)	0.250 ± 0.070(b)

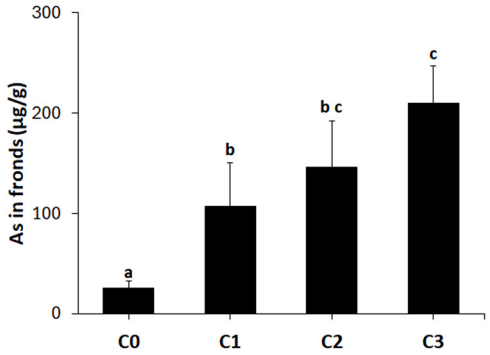

Fig. 4. Analysis of the amount of As in fronds over time during the 3 subsequent phytofiltration cycles (C0-C3) using multiple ferns. Data is presented as mean ± SE (n = 2). Different letters indicate significant differences ($P < 0.05$) among different sampling timepoints at each phytofiltration cycle, respectively.

successive reuse cycles, can be mechanistically explained by the increased age and biomass causing more efficient As uptake.

Notably, this efficiency was maintained under higher planting densities, demonstrating the scalability of this approach. Altogether, the study provides evidence that *P. vittata* is not only a hyperaccumulator but also a robust and reusable "biological filter," paving the way for its application as environmentally sustainable water treatment systems.

CRediT authorship contribution statement

Enrico Veschetti: Writing – review & editing. **Luca Lucentini:** Writing – review & editing. **Maura Cardarelli:** Writing – review & editing, Supervision, Conceptualization. **Patrizia Brunetti:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Davide Marzi:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Clara Sette:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Lorenzo Massimi:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Alice Zara:** Methodology, Investigation, Formal analysis. **Maria Luisa Antenozio:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation.

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Declaration of Competing Interest

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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.eti.2025.104629.

Data Availability

All data supporting the results are included within the paper and its supplementary materials

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