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Advanced Drinking Groundwater As Phytofiltration by the Hyperaccumulating Fern *Pteris vittata*

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Abstract: The reuse of *Pteris vittata* plants for multiple phytofiltration cycles is a main issue to allow an efficient phytoremediation of arsenic (As)-contaminated groundwater. Here, we assessed the capacity of phytofiltration of *P. vittata* plants grown for two cycles on naturally As-contaminated drinking water (collected in Central Italy), spaced by a growth cycle on non-contaminated water (N cycle). *P. vittata* young plants, with extensive frond and root development, were suspended individually in 15 L of water with initial As of 59 µg/L, without any additional treatment or water refilling. During cycle 1, in 45 days *P. vittata* plants reduced As concentration below 10 µg/L, the allowed EU limits for drinking water. During the subsequent 30 day-N cycle on non-contaminated water, no leaching of As from the roots was observed, while the water pH increased 0.9 Units, but is within the allowed limits. During cycle 2, under the same conditions as cycle 1, As concentration decreased below 10 µg/L in less than seven days. These results show that *P. vittata* young plants, previously used for the phytofiltration of As, do not extrude As and, when reused, remove As much more rapidly. No additional treatments were required during phytofiltration and thus this represents a sustainable, efficient, and scalable strategy.

Keywords: arsenic; drinking water; phytofiltration; *Pteris vittata*

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1. Introduction

Arsenic (As) is a toxic carcinogenic metalloid ubiquitously distributed in the environment [1]. Arsenic contamination could be due to natural geological events or to human industrial or agricultural activities and could affect both soil and water quality [2]. Arsenic contamination of drinking and irrigation water is an important threat to public health [3,4] and current European legislation imposes a limit of 10 µg/L for As concentration in water for human consumption. In several volcanic areas this limit is highly exceeded because of natural mineral release from soil to underground aquifers [5–7]. As a consequence of the excess of As concentration, in Viterbo and the surrounding area (Lazio, Italy), de-arsenification of drinking water is performed by means of filtering devices. In particular, the most used filtration systems used in this area are based on iron oxide adsorbents and constitute a high cost for the community (EGATO1-Vt sources). In fact, these absorbents work more efficiently in an acid environment and therefore water pre-treatment is required; in addition, adsorbents should be replaced cyclically. An alternative adsorption method could be the activated alumina, which is easy to manage and relatively low cost for the removal of arsenic and fluoride ions from water [8,9]. The downside is the possible release of aluminium into water.

Phytoremediation techniques, i.e., the use of plants to clean contaminated water and soils are gaining an increasing interest for As remediation of drinking water. Indeed, this environmentally sustainable strategy could be a valid solution upstream from the use of filters.

The fern *Pteris vittata* is a hyperaccumulator, which is capable of taking up and accumulating in fronds up to 22.000 mg/Kg As from the soil and thus can be used to clean up As-contaminated soils [10].

Increasing evidence suggests that *P. vittata* is able to grow hydroponically and to accumulate As when grown on contaminated water [11–14]. In addition, it was shown that *P. vittata* is more efficient than the non-hyperaccumulator fern *Nephrolepis exaltata* [15].

Hydroponical growth of *P. vittata* plants allows the development of the root system that is essential for As uptake [16–19]. It has been shown that adventitious and young roots, located in the first 10–20 cm of the underground tissue, seem to be responsible for active absorption in water, using low strength nutrition solution [12,14]. Most of these studies were conducted by growing *P. vittata* on water supplemented with As salts, or on As-contaminated water supplemented with other elements such as P and N [15,20–22]. In contrast, few hydroponic experiments were performed on water without further additions; in this case, water collected in Florida was probably anthropically polluted by the use of pesticides [17,18].

Limited information is available on possible effects of *P. vittata* growth on water pH. It was shown that water pH could change after *P. vittata* growth, as Stamps et al. [14] showed an increased pH value in hydroponic culture medium. However, no measurements were done on water pH after phytofiltration by *P. vittata.* This is a major issue for using phytofiltration on water for human consumption, as the pH value cannot exceed the allowed limits of 9.5 (98/83/CE).

Different groups investigated the possibility of reusing the same plants in multiple cycles and obtained opposite results: A decrease in As uptake during the second cycle was shown by Tu et al. [23] and Huang et al. [15], whereas an increased in As uptake during subsequent cycles was shown by Natarajan et al. [17,18,24].

The objective of this study, by utilizing As-contaminated drinking water collected in Nepi, a town near Viterbo (Lazio, Italy), was to evaluate: (i) The efficiency of *P. vittata* young plants to remove As during cycle 1; (ii) the release of As from *P. vittata* roots, and possible changes in pH values during the subsequent N cycle (on non-contaminated water); (iii) the rapidity of As uptake when the same *P. vittata* plants are reused for cycle 2.

2. Materials and Methods

2.1. Arsenic-Contaminated Groundwater

Naturally contaminated groundwater for the experiments, kindly provided by EGATO1-Vt, and Talete Spa (https://www.taletespa.eu/ (accessed on 30 May 2019)), was collected from Varano waterwell, located in Nepi (Viterbo, Lazio, Italy, 42°15′29.2″ N 12°18′24.2″ E, Figure 1) and was stored in polyethylene tanks at room temperature. Groundwater was collected twice and the As concentration was, respectively, 59.7 µg/L and 46.04 µg/L, while pH was 7.3 in both.

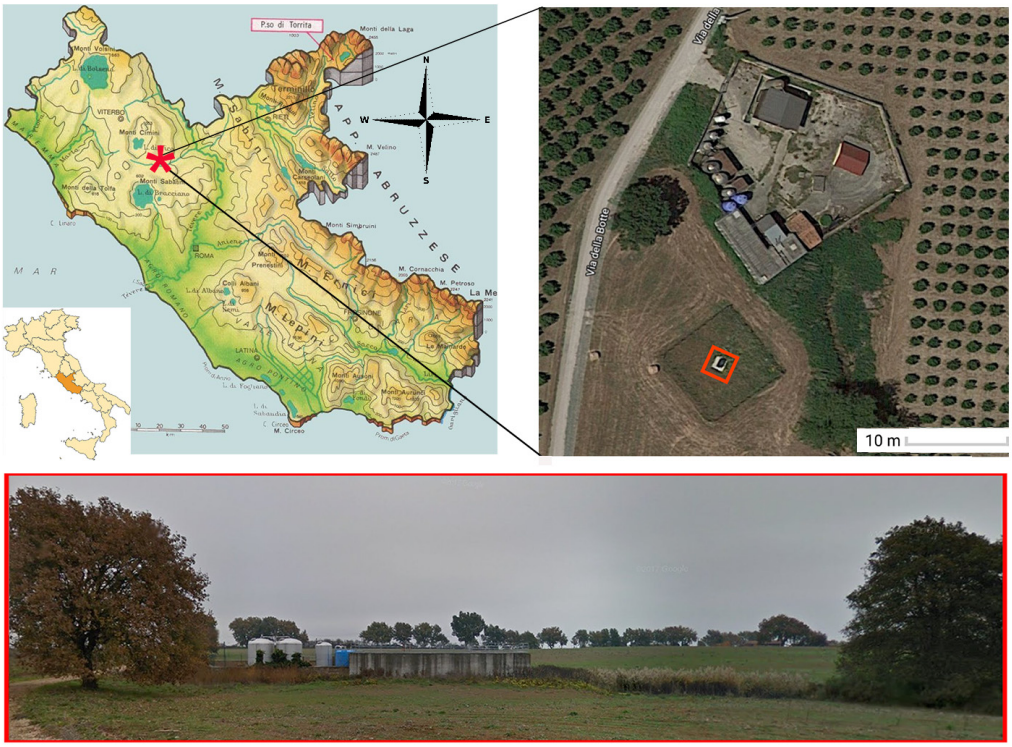

Figure 1. Varano waterwell location where water was collected for this study. A detailed map of the Lazio region is shown and the specific area is indicated by a star. The red rectangle on the enlarged image indicates Varano waterwell.

2.2. Plant Growth

A Chinese fern brake (*Pteris vittata*) collected from the Botanical Garden of La Tuscia University (Viterbo, Italy) was moved to a thermostated greenhouse for propagation [25]. In addition to natural sunlight irradiation, greenhouse illumination was supplied with 100 µmol/m^{2}s white light lamps set to a photoperiod of 16 h light and 8 h dark. Greenhouse temperature and humidity were set to 28 °C and 70%, respectively. Mature spores were collected from fertile fronds, resuspended in water and sown in pots filled with soil and expanded clay and covered with a thin plastic film. Spores germinated and gametophytes arose after 4 weeks (Figure S1). The reproduction process occurred quickly and 4 weeks later the young sporophytes reached a height of about 2 cm, showing at least two small fronds. Young sporophytes were moved into individual pots with a soil mixture (pet/perlite/expanded clay) for about 8–10 weeks, without adding fertilizers. At this stage, all the sporophytes showed 4–6 fully developed leaves and an expanded radical system, and thus were moved to the hydroponic system.

Here, mature sporophytes were supplied with fertilizers and water electroconductivity (EC) was set to 2 (mS/cm), while water pH was naturally established at 8.4 by ferns themselves. In this condition, ferns needed about 4 weeks of hydroponic growth to be considered ready for subsequent experiments. EC values were also monitored during *P. vittata* growth on non-contaminated water with a portable Conductivity Meter (Milwaukee EC60 model, (Milwaukee Instruments, Inc, Szeged, Hungary) to check the mineral content of the water. Results show a stable EC during the first 14 days of growth on contaminated water with a slight but significant decrease after 2 days (Figure S2).

2.3. Hydroponic Growth Systems

For plant growth and phytofiltration experiments, we adopted two hydroponic growth systems (https://www.idroponica.it/ (accessed on 9 August 2021)):

- A Deep Water Culture (DWC) hydroponic system, based on a 15 L bucket housing a single fern and equipped with an air pump to oxygenate water (Figure 2A,B). This system was used for phytofiltration experiments.
- A Recirculating Deep Water Culture (RDWC) hydroponic system, composed by 4 individual deep water culture systems (DWC) connected together and with a central control tank that allows the simultaneous control of pH and nutrients (Figure 3A,B). The control tank is equipped with a water pump that moves the water constantly from the tank to each bucket (outward water flow) and back to the control tank (inward water flow); each bucket contains an air pump that increases water oxygenation. This system was used for sporophytes growth, prior to experiments.

Figure 2. Deep Water Culture (DWC) system. (**A**) a single bucket with an independent oxygenation. (**B**) Functionl Scheme of the DWC.

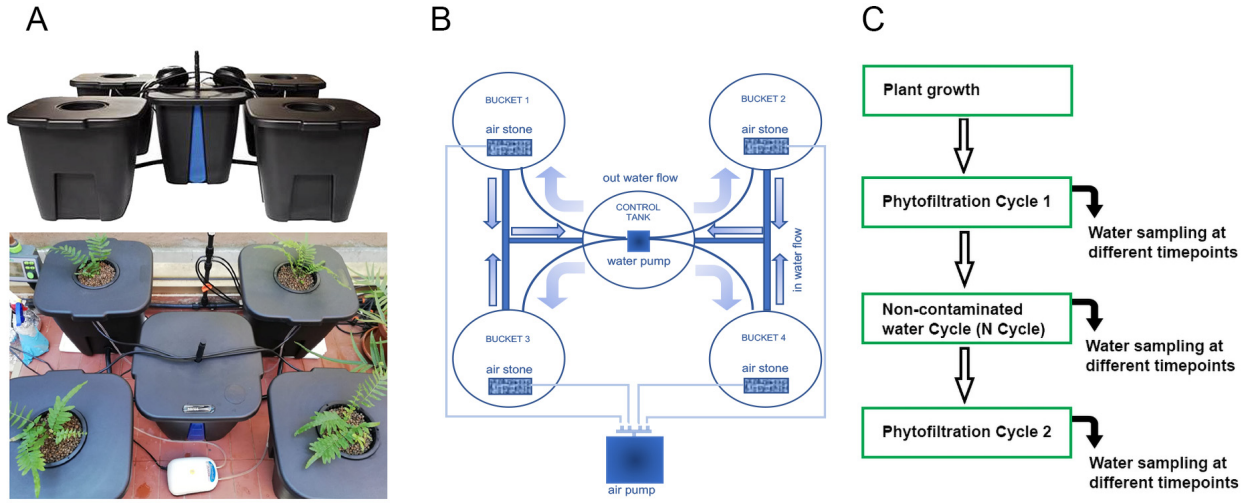

Figure 3. Recirculating Deep Water Culture (RDWC). (**A**) RDWC system used for sporophytes growth, showing a central control tank connected to 4 DWC buckets. (**B**) Functionl Scheme of the RDWC, indicating the components of the system and the outward water flow and inward water flow, respectively from and to the control tank. (**C**) Schematic workflow of phytofiltration experiments, performed under environmental controlled conditions, using As contaminated water collected from Varano waterwell. out water flow: outward water flow, in water flow: inward water flow.

2.4. Phytofiltration Experimental Set Up

A schematic workflow of phytofiltration experiments is shown in Figure 3C: Each fern was moved to a Deep Water Culture (DWC) hydroponic system composed by individual buckets provided with air pumps and filled with 15 L of naturally As-contaminated groundwater (Figure 3B,C, Supplementary Figure S1). No pre-treatment to adjust the pH conditions, neither fertilizers were added during the experiments. Evapotranspiration (ET) was evaluated and it was chosen not to be compensated. At the end of the first phytofiltration cycle, ferns were moved to uncontaminated and unfertilized water for 30 days for further analysis. Next, the same ferns were re-used for a second phytofiltration cycle, using a second batch of naturally As-contaminated groundwater.

2.5. Chemical Analysis

Water samples (50 mL) were collected before and during phytofiltration cycles at different times. They were then acidified to 1% with fuming nitric acid, and analyzed with a Perkin–Elmer NexION 300D ICP mass spectrometer (PerkinElmer Inc., Shelton, CT, USA) operating with an ESI autosampler (PerkinElmer Inc., Waltham, MA, USA) and an ultrasonic nebulizer (Teledyne Cetac, Omha, NE, USA) U6000AT+ Teledyne Cetac Technologies. ICP-MS operating conditions (that is, argon flowrates into nebulization chamber and torch for sample nebulization and plasma generation and containment as well as plasma generation potential, 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 mg/L for each element and 1% of HNO_3. Quantitative determination of As was performed operating under standard mode conditions, without activating the collision/reaction cell, as the polyatomic interference ($^{40}Ar^{35}Cl^{+}$) 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 (HNO_3 1%), five external standard solutions in the range of 1–50 µg/L and 20 µg/L ^{115}Indium internal standard, the latter added to samples and external standards by peristaltic pump. Every standard solution was prepared by diluting the corresponding 1000-mg/L solution (AccuTrace Reference Standard) and acidified with extra-pure fuming nitric acid.

3. Results and Discussion

3.1. Hydroponic Growth Systems

To monitor the concentration of As in contaminated water over time, in the absence of *P. vittata* plants and in the presence of an air pump, preliminary analysis have been conducted. Samples of water at T0, 15 days and 30 days were collected and As concentration measured by ICP-MS. As shown in Supplementary Table S1 there was only a negligible decrease in As concentration over time.

Young ferns used for phytofiltration experiments were initially grown 4 months in soil and subsequently 4 weeks in the RDWC hydroponic system (Figure S1, Figure 3A,B), which allows to control and monitor ferns growth, exposing plants to the same water pH, nutrient density and oxygenation.

This ensures a more homogenous growth for all plants providing optimal conditions for phytofiltration experiments. Furthermore, the RDWC system is easily scalable. Previous works have shown that the fern growth stage, defined as the number and age of the fronds and the length of the root system, is the most critical parameter for an efficient phytofiltration of As [12,15,17,18,23,24]. Hence in this study we focused on the homogeneity of plant growth prior to experiments, in order to increase the efficiency of phytofiltration cycles and obtain reproducible data.

3.2. Phytofiltration Cycle 1

Three young ferns (#1, #2, #3) which showed 6–8 fronds and a 20–25 cm long root system were chosen for the first cycle (cycle 1). Ferns #1, #2 and #3 were placed in single pots, filled with 15 L of naturally As-contaminated groundwater with an initial concentration of 59.705 µg/L and aerated by individual air pumps (DWC system, Figures 2A,B and 4A). Water sampling was performed at different times and As concentration measured by ICP-MS. As shown in Figure 4B, ferns were able to reduce As concentration about 15%, 20%, 50% and 98%, in 24 h, 48 h, 30 days and 60 days, respectively. These results are in good agreement with the experiments conducted by Natarajan et al. [17,24] with hydroponically grown ferns, under similar phytofiltration conditions. The authors transferred two ferns per tank in 30 L of As-contaminated water (130 µg/L As) amended with modified 0.25 strength Hoagland's solution and found that ferns needed 5 weeks to decrease As concentration below 10 µg/L. In contrast, further experiments performed by the authors used completely different phytofiltration conditions, such as 8 plants per tank in 45 L [18], while other authors added growth elements to contaminated water [15,22]. Our results suggest that young ferns grown in soil and subsequently for a short period in a low-nutrient RDWC hydroponic system, when transferred to naturally As-contaminated ground water, show an arsenic uptake similar to that obtained with hydroponically grown plants supplied with nutrients.

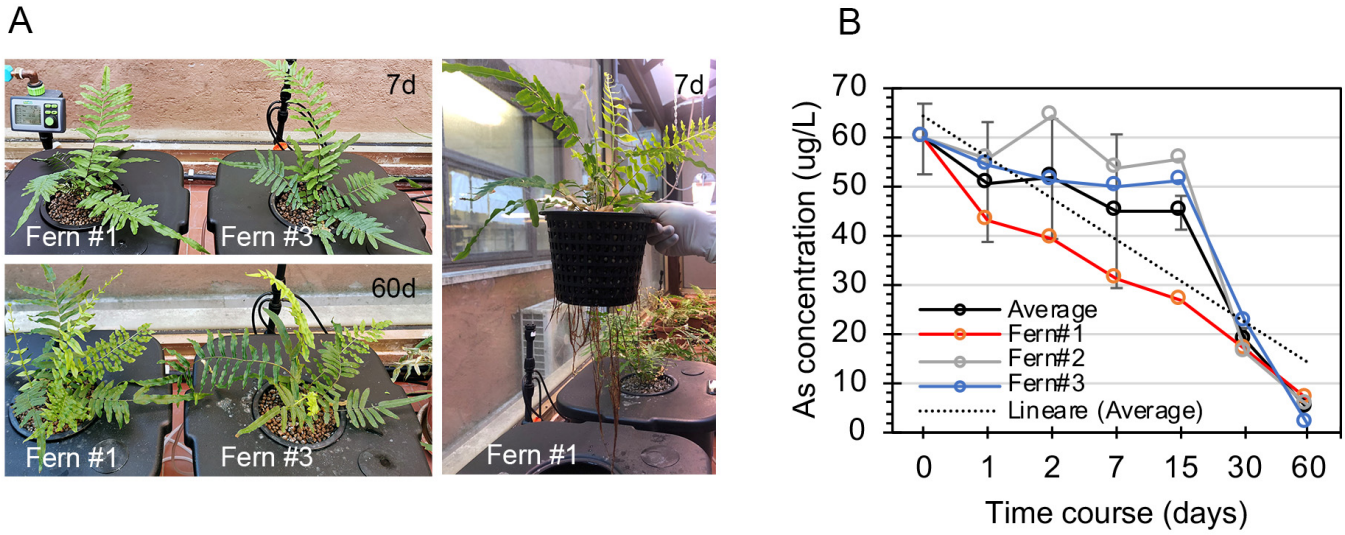

Figure 4. Phytofiltration experiment cycle 1. (**A**) Two Ferns in individual buckets filled with 15 L of naturally As-contaminated groundwater after seven days (upper panel) and 60 days (lower panel). The fronds and roots of Fern 1 after seven days on As treatment (right panel). (**B**) The amount of arsenic in the water at different timepoints is reported for each fern at the indicated times: Fern #1 red line; Fern #2 grey line; Fern #3 blue line. Average value ±SD is indicated by the black line; linear average dashed line.

3.3. Arsenic Excretion and pH Changes during N Cycle Performed after Phytofiltration Cycle 1

After cycle 1, ferns were moved to buckets filled with non-contaminated water to evaluate two parameters: (i) Possible excretion of the accumulated As, (ii) water pH after *P. vittata* growth.

Several papers reported an efflux of arsenite and arsenate from root cells to the external medium, in different plant species, while little is known about As efflux from *Pteris vittata* [26,27]. Thus, to evaluate whether ferns used in cycle 1 could excrete the accumulated As, ferns #1, #2 and #3 were grown for one month in single pots, filled with 15 L of non-contaminated water and aerated by individual air pumps (DWC system, Figure 2A,B) and water sampling was performed at 0, 7, 15 and 30 days. As shown in Figure 5A, As concentration remains below 3.2 µg/L in all samples with no increase

over time. These results show that ferns do not excrete or excrete negligible amounts of accumulated As.

As *P. vittata* is able to secrete hydroxide ions (OH^-) and modifies pH values when grown in soils [28], we evaluated if this could also happen when ferns are grown in water. Thus, we measured water pH at different times, 0, 2, 4, 10 and 14 days. As shown in Figure 5B water pH increases from 7.3 to 8.4 in four days without any further subsequent increase. This is consistent with previous studies [14], which showed that *P. vittata* grown at different nutrient concentrations is able to modify and increase the pH value above 8 in hydroponic cultures. The limited increase in the pH value and the negligible release of As, following *P. vittata* growth, indicate that the ferns can be reused for subsequent phytofiltration cycles.

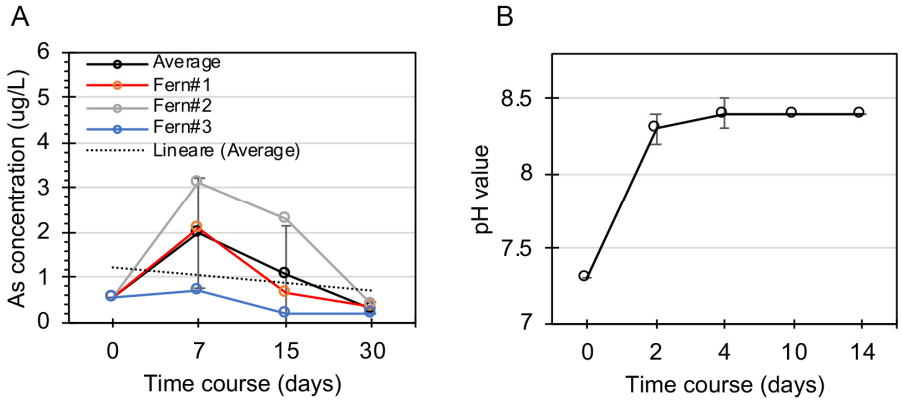

Figure 5. Arsenic excretion and pH changes during N cycle after phytofiltration cycle 1. **(A)** Evaluation of As excretion by ferns. As concentration in water was measured at different timepoints, from 1 to 60 days. Fern #1 red line; Fern #2 grey line; Fern #3 blue line; average black line; (**B**) linear average dashed line pH was monitored by subsequent water samplings. Ferns #1–3, grown in individual buckets, were used and the average of samplings for each timepoint is reported. Error bars are means ± SD.

3.4. Phytofiltration Cycle 2

Conflicting results were obtained on the efficiency of As uptake for ferns reused in multiple phytofiltration cycles [15,18–24]. To investigate the capacity of reused ferns to uptake As, we used the same plants to perform a second phytofiltration cycle (cycle 2), under the same conditions as for cycle 1. At this time, ferns showed 10–12 fronds and a 30–35 cm long and expanded root system (Figure 6A). Ferns #1, #2 and #3 were placed in single pots, filled with 15 L of naturally As-contaminated groundwater and aerated by individual air pumps (DWC system, Figures 2A,B and 6A,B). As shown in Figure 6B, ferns during cycle 2 removed 99.5% of As in less than seven days, indicating an increase in As uptake of about 45-fold, from cycle 1 to cycle 2. These results show that reused ferns are more efficient in As removal from natural contaminated water, in agreement with Natarajan [18–24], who reused ferns on water samples contaminated by pesticides. The increase in As uptake showed by reused ferns is possibly due to a larger size of the aerial and root system [23] or could be related to an increased capacity of As uptake due to the previous growth in the presence of As. In addition, our results show that to achieve an efficient and fast dearsenification for drinking water, the homogeneity of plant growth is an additional parameter to consider. This can be improved through advanced systems such as RDWC as well as the use of micro and macro elements before plants transfer to As-contaminated water.

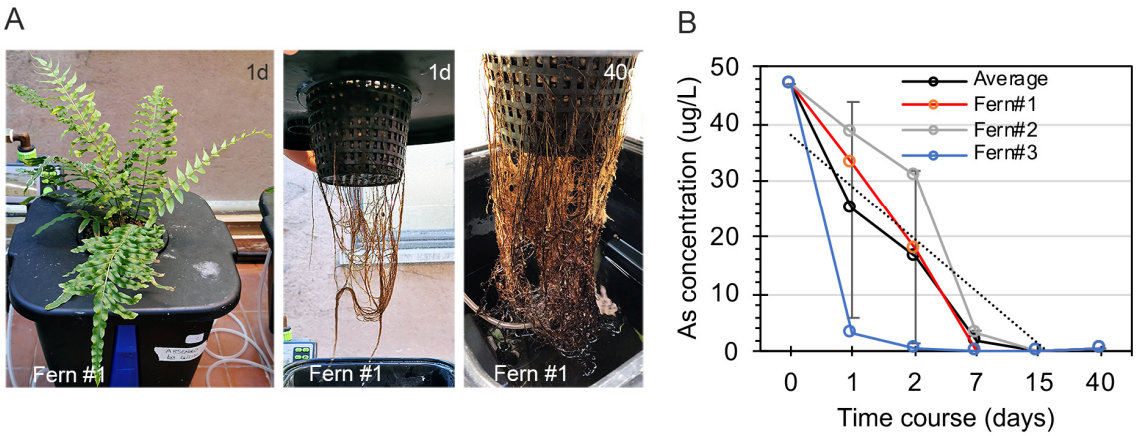

Figure 6. Phytofiltration experiment cycle 2. (**A**) Ferns from cycle 1 were placed in individual buckets filled with 15 L of naturally As-contaminated groundwater. Fern 1 is shown after one day (left panel) and the fronds and roots of Fern 1 after one day (central panel) and 40 days (right panel) on As treatment. (**B**) The amount of arsenic in the water at different timepoints is reported for each fern at the indicated times from one day to 40 days: Fern #1 red line; Fern #2 grey line; Fern #3 blue line. Average value ±SD is indicated by the black line.

4. Conclusions

Here we show that young *P. vittata* plants, grown in soil and subsequently for a short period in in a low-nutrient RDWC hydroponic, can be used for at least two cycles, resulting in a faster As uptake during the second cycle. Furthermore, we demonstrate that *P. vittata*, when transferred into non-contaminated water after cycle 1, does not excrete the absorbed As but causes a limited increase in water pH, possibly due to OH^- excretion. Altogether our results indicate that *P. vittata* phytofiltration is a promising technology to remove As from drinking water. Future studies aimed at optimizing *P. vittata* growth conditions will be necessary to increase initial efficiency in phytofiltration and allow the fern reuse in multiple consecutive cycles.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/w13162187/s1, Figure S1: Stages of fern development from spore germination to young sporophytes ready for phytofiltration. A timeline describes the weeks needed for each growth phase, Figure S2: Electrical Conductivity (EC) measurement. 12 ferns were grown in 15 -litre of water without adding any nutrients. The EC value was evaluated at different time point (T0, T2, T4, T10, T14). The experiment was performed in triplicate. Statistical differences compared to T0 were evaluated by student's *t*-test (* $p < 0.5$), Table S1: Arsenic concentration in water was measured at T0 and after 15 and 30 days, in the absence of ferns and in the presence of an air pump, as a preliminary control.

Author Contributions: P.B., M.C. and D.M. contributed to the conceptualization and design, to the methodology and investigation. Plant propagation, hydroponics preparation, water samples collection were performed by D.M., M.L.A. and S.V. Water samples analysis by ICP-MS, was performed by C.S. under the supervision of E.V. and L.L. Data analysis was performed by P.B., M.C., D.M., E.V., L.L., G.D. provided sample waters and map of the site. The original draft of the manuscript was written by P.B., D.M. and M.C. and the final version was supervised by Maura Cardarelli. All authors commented on previous versions of the manuscript. All authors have read and agreed to the published version of the manuscript.

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