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Enhanced Effect of Phytoextraction on Arsenic-Contaminated Soil by Microbial Reduction

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Abstract: The gradually increasing presence of arsenic, a highly toxic heavy metal, poses a significant threat to both soil environmental safety and human health. *Pteris vittata* has long been recognized as an efficient hyperaccumulator plant for arsenic pollution. However, the pattern of arsenic accumulation in soil impacts its bioavailability and restricts the extraction efficiency of *Pteris vittata*. To address this issue, microorganisms have the potential to improve the arsenic accumulation efficiency of *Pteris vittata*. In this work, we employed anthropogenic enrichment methods to extract functional iron–sulfur-reducing bacteria from soil as a raw material. These bacteria were then utilized to assist *Pteris vittata* in the phytoremediation of arsenic-contaminated soil. Furthermore, the utilization of organic fertilizer produced from fermented crop straw significantly boosted the remediation effect. This led to an increase in the accumulation efficiency of arsenic by *Pteris vittata* by 87.56%, while simultaneously reducing the content of available arsenic in the soil by 98.36%. Finally, the experimental phenomena were studied through a soil-microbial batch leaching test and plant potting test. And the mechanism of the microorganism-catalyzed soil iron–sulfur geochemical cycle on arsenic release and transformation in soil as well as the extraction effect of *Pteris vittata* were systematically investigated using ICP, BCR sequential extraction and XPS analysis. The results demonstrated that using iron–sulfur-reducing microorganisms to enhance the phytoremediation effect is an effective strategy in the field of ecological restoration.

Keywords: arsenic-contaminated soil; phytoremediation; microbial reduction; *Pteris vittata*; iron-sulfur-reducing bacteria

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1. Introduction

Arsenic is a highly toxic heavy metal. The accumulation of arsenic in soil causes serious environmental problems including damaging soil structure, poisoning crops and animals and killing native microbiota [1,2]. For human beings, prolonged exposure to arsenic-contaminated environments may cause skin cancer or disrupt biological systems, putting additional stress on the kidneys, liver, lungs and other essential organs [3,4]. According to a report by the Chinese State Environmental Protection Administration, arsenic pollution has become an urgent and widespread problem for southwest China [5], and efficient ecological restoration technology for soil arsenic contamination is sorely needed [6].

Phytoremediation is an environmental pollution remediation method that has emerged in recent years which is not only widely applied in soil remediation but also finds applications in water and air pollution control [7,8]. Phytoremediation, including phytoextraction, phytostabilization and phytofiltration, emerged as an environmentally friendly approach for soil remediation during the 1990s [9,10]. This plant-based method employs processes

such as translocation, accumulation, transport, transformation and volatilization to remove arsenic from soil [11]. Since inorganic arsenic interferes with plant metabolic processes and inhibits plant growth, phytoremediation requires plants with high arsenic resistance [12]. Earlier, researchers used Carex rostrata, Eriophorum, angustifolium, Phragmites, etc., for phytoremediation studies of arsenic-contaminated soils [13]; the results were unsatisfactory until *Pteris vittata*, an arsenic hyperaccumulator, was discovered. The roots of *Pteris vittata* have a strong absorption capacity for arsenic, and its rhizomes, which have a unique set of protective mechanisms to guard against the toxic effects of high concentrations of arsenic, can efficiently translocate arsenic [14]. As a result, *Pteris vittata* has become a reliable candidate for the phytoremediation of arsenic-contaminated soil [15].

The effectiveness of phytoremediation towards arsenic is influenced by various factors. Only soluble arsenic can be absorbed by the roots of *Pteris vittata* during the remediation process, and thus the fractionation of arsenic in the soil plays a significant role. The mobility and bioavailability of arsenic in soil can be altered by microorganisms through a variety of reactions, including adsorption, oxidation and reduction, complexation, transformation and volatilization [16]. Therefore, many studies focus on the microorganisms which could assist *Pteris vittata* in the remediation of arsenic-containing soil. And it has been found that the growth of Pteris vittate maintains a dynamic equilibrium relationship with the variety of microbial communities in the rhizosphere as well as the speciation and level of arsenic in the rhizospheric soil [17]. Therefore, introducing functional strains exogenously may trigger changes in the rhizosphere's microbial community structure, leading to alterations in arsenic release, speciation, *Pteris vittata* biomass and arsenic accumulation. For instance, Li et al. [18] discovered that the arsenic accumulated by *Pteris vittata* was increased by an Enterobacter sp. E1 strain, which enhanced the desorption and reduction of arsenic in the iron-hydroxide-bound state. Zhu et al. [19] demonstrated that arsenate-reducing bacterial inoculants and composite microbial inoculants increased dissolved organic carbon and available arsenic in the soil, stimulating the growth of *Pteris vittata* and arsenic accumulation.

Unlike the aforementioned study, this research not only focuses on the release and reduction processes of arsenic, but also examines the influence of microorganisms on sulfur and iron elements in the soil, as well as their counteractive effects on the transformation of arsenic. Numerous studies have shown that the release, migration and transformation of arsenic are closely related to organic matter [20,21] and iron–sulfur geochemical cycling processes in anaerobic environments involving soil and groundwater [22]. For example, bacterial-accelerated oxidative acidification of sulfides leads to the release of arsenic by mineral dissolution [23]. And bacterial-induced reductive dissolution of iron oxides causes the release and reduction of arsenic in the adsorbed state [24]. Therefore, in this work, functional iron–sulfur-reducing flora was enriched. The advantage of microbial flora over single strains in soil remediation lies in their ability to synergistically facilitate the translocation of pollutants from the soil to the aboveground parts of plants, as well as the accumulation, transformation and utilization of these pollutants within the plant body, thereby effectively facilitating the remediation of environmental pollution. Additionally, plant organic matter from straw fermentation was utilized as a carbon source for microorganisms and as nutrients for *Pteris vittata*. Ultimately, a novel microbial-*Pteris vittata* remediation mechanism centered around biocatalytic iron–sulfur reduction and arsenic speciation transformation was proposed.

2. Materials and Methods

2.1. Materials Preparation

The arsenic-contaminated soil was collected from a farmland 100 m away from southeast of the Yongfeng Chemical Metallurgical Plant in Huilong Town, Zhongshan County, Hezhou City, Guangxi Zhuang Autonomous Region. The arsenic contamination in the farmland was mainly due to smoke and dust from the plant's production. The soil was

collected from 0–20 cm of the surface layer of the farmland and stored in a refrigerator at 4 °C. The soil characteristics are shown in Table 1.

Table 1. Physicochemical properties of raw soil.

Properties	Value
pH	6.98 ± 0.04
cation exchange capacity (cmol/kg)	14.54 ± 0.53
Organic carbon (g/kg)	20.43 ± 2.16
Total nitrogen (g/kg)	1.56 ± 0.13
Available nitrogen (mg/kg)	193.22 ± 17.86
Total phosphorous (g/kg)	2.18 ± 0.21
Available phosphorous (mg/kg)	85.35 ± 13.64
As(mg/kg)	265.48 ± 23.36
Available As(mg/kg)	16.43 ± 2.38

2.2. Isolation of Iron-Sulfur-Reducing Bacteria

The iron–sulfur-reducing bacteria were isolated from sediment samples collected from contaminated farmland puddles. The sediment samples were carefully collected and transported back to the laboratory in sealed bags. Upon arrival, they were stored in a refrigerator at −20 °C. To create a microculture system, a batch of 250 mL screw-capped sealed serum bottles was prepared. The medium was prepared according to the specifications outlined in Table 2. The sediment and medium were then loaded into the sealed serum bottles at a solid–liquid ratio of 1:10 g/mL. The bottles were filled with nitrogen to establish an anaerobic environment. The bottles were incubated in a constant-shaking incubator at a temperature of 35 °C for a period of 5–7 days. When the sediment turned black, the upper suspension in the serum bottle was inoculated into a new medium to continue incubation. This step was repeated 2–3 times.

Table 2. Components of bacterial culture media.

KH_2PO_4	K_2HPO_4	$MgCl_2$	$CaCl_2 \cdot 2H_2O$	Na_2SO_4	$Fe_2(SO_4)_3$	Yeast Extract	$C_3H_5NaO_3$
0.5 g/L	1 g/L	0.25 g/L	0.1 g/L	1.2 g/L	0.5 g/L	1 g/L	0.5 g/L

2.3. Preparation of Plant Organic Matter (POM)

Rice and corn straw, which are the representative crops of Guangxi, were used as the source of organic matter for the iron–sulfur-reducing bacteria. The straw was air-dried and crushed, water was added and stirred to make the moisture content reach 50% and a small dosage of lactobacilli was added. Anaerobically fermenting the mixture at 30 °C for 15 days produced plant organic fertilizers; then, after evenly spreading the organic fertilizer on the clean bench, the ultraviolet light irradiation was turned on for ten minutes in order to inactivate the fertilizer's bacteria and lessen the interference on the iron–sulfur-reduction bacteria. The basic organic matter content of the fermented straw is shown in Table 3.

Table 3. Physicochemical properties of POM.

pH	Lactic Acid	Acetic Acid	Fiber	Protein
4.52	32.48 g/kg	8.26 g/kg	38.43%	4.28%

2.4. Soil-Microbial Batch Tests

The air-dried and sieved soil was mixed with deionized water, iron–sulfur-reducing bacteria and plant organic matter. This mixture was then placed in 150 mL screw-cork serum bottles, which were filled with nitrogen to create an anaerobic environment. The batch leaching test was performed in the following groups:

Control group: 10 g of soil mixed with 100 mL of deionized water.

Bacteria group: 10 g of soil mixed with 90 mL of deionized water and 10 mL of bacterial sap.

Bacteria + POM group: 10 g of soil mixed with 90 mL of deionized water, 10 mL of bacterial flora and 5 g of plant organic matter.

The batch tests were conducted in a thermostatic shaking incubator at a temperature of 30 °C and a speed of 180 rpm for a duration of 7 days. The supernatant was collected daily for examination.

2.5. Plant Potting Experiment

Before the experiment, the soil was air-dried under a natural environment and then sieved through a 2 mm nylon sieve to remove stones and plant rhizomes and other debris. Then, the soil, along with the plant organic matter and iron–sulfur-reducing bacterial solution, was distributed according to Table 4, resulting in a total of five treatment groups. The *Pteris vittata* seedlings were transplanted into pots with a diameter of 16 cm and a height of 14 cm. These pots were then placed in an artificial climatic incubator and incubated for four months, with a light exposure of 12 h per day and a temperature of 30 °C and 60% humidity. At the end of the period, the rhizosphere soil of *Pteris vittata* was taken to detect the fractionation of arsenic by the BCR sequential extraction method, and the available arsenic was extracted from the soil with 0.5 mol/L $NaHCO_3$.

Table 4. Experimental grouping of *Pteris vittata* in pots.

Group	As Contaminated Soil	*Pteris vittata*	Microbial Flora	POM
T1	1 kg	1 plant	/	/
T2	1 kg	/	50 mL	/
T3	1 kg	1 plant	50 mL	/
T4	1 kg	/	50 mL	50 g
T5	1 kg	1 plant	50 mL	50 g

Treatment Group T1: Established as a blank control group to investigate the extraction capability of *Pteris vittata* on soil heavy metals without the addition of iron–sulfur-reducing bacteria and plant organic matter.

Treatment Group T2: *Pteris vittata* was not planted; only iron–sulfur-reducing bacteria liquid was inoculated into the soil to examine the role of bacteria in the transformation of soil heavy metals into their available forms and fractionation.

Treatment Group T3: To compare with T1 and observe whether inoculation with iron–sulfur-reducing bacteria can enhance the absorption of heavy metals by *Pteris vittata*.

Treatment Group T4: In contrast to T2, *Pteris vittata* was not planted; only bacteria were inoculated, and plant organic matter was added to explore if the addition of organic matter benefits the redistribution of heavy metals by bacteria and assess the impact of bacteria and plant organic matter on the speciation of heavy metals in the absence of *Pteris vittata* participation.

Treatment Group T5: In comparison with T1 and T3, to observe whether the addition of plant organic matter can further promote the remediation effectiveness of *Pteris vittata*.

Pteris vittata plants were split into roots and fronds for plant samples, washed with deionized water, dried at 70 °C for 24 h and weighed to record the biomass. All soil and plant samples were digested in a graphite digestion furnace with 10 mL of 50% HNO_3 and 2 mL of 30% H_2O_2 for total arsenic analysis, and arsenic concentrations of the digested samples were determined by inductively coupled plasma emission spectrometry (ICP-OES, Optima 5300DV, Perkin Elmer, Waltham, MA, USA).

2.6. Analysis of Arsenic Speciation in Pteris vittata

Arsenic was extracted by ultrasonic extraction using a 1:1 aqueous solution of methanol, and the extraction was repeated twice at 60 °C for 4 h. To determine the arsenic content

of the samples, inductively coupled plasma emission spectrometry coupled with a hydride generator (ICP-OES-HG) was used. The samples were divided into two batches: one batch had thiourea added in advance to determine the total arsenic concentration (as thiourea reduces all pentavalent arsenic in solution to trivalent arsenic). The other batch did not have thiourea added and was used to determine the trivalent arsenic concentration. The pentavalent arsenic concentration was calculated by subtracting the trivalent arsenic concentration from the total arsenic concentration.

2.7. Microbial Community Structure Analysis

Fresh rhizosphere soil samples collected after four months of phytoremediation were subjected to analysis of the microbial community structure. Genomic DNA was used as the template for PCR amplification and product electrophoresis. Primers with barcode primers and Premix Taq (TaKaRa) were employed for PCR amplification, with the selection of sequencing regions based on the experiment's requirements. The PCR products were mixed using Gene Tools Analysis Software (Version 4.03.05.0, SynGene, Cambridge, UK) after comparing their concentrations and calculating the required volume for each sample, following the principle of equal mass. The mixed PCR products were then recovered using the E.Z.N.A. Gel Extraction Kit, and the target DNA fragments were eluted in TE buffer. Subsequent library construction was conducted following the standard procedure of the NEB Next Ultra DNA Library Prep Kit for Illumina. Once completed, sequencing was performed on the Miseq PE300/Nova Seq PE250 (Illumina, San Diego, CA, USA) high-throughput sequencing platform. The raw image data obtained from the sequencing process were subjected to Base Calling analysis, resulting in the transformation into Raw Reads.

2.8. Data Analysis

The experimental data were processed using Excel2021, XPS Peak41 and Avantage599-31 and plotted using Origin 2023 and Power Point2021. Data are presented as the mean of three replicates with standard errors.

3. Results and Discussion

3.1. Composition of Iron-Sulfur-Reducing Flora

The iron–sulfur-reducing flora obtained through artificial enrichment was dominated by Desulfovibrio, Escherichia-Shigella, Paraburholderia and Achromobater, as depicted in Figure 1. Among them, Desulfovibrio, an acknowledged sulfate-reducing bacteria (SRB), had strong sulfate-reducing ability [25] and exhibited the highest relative abundance, suggesting that enrichment was achieved. Desulfovibrio plays an irreplaceable role in the biotransformation of sulfate by converting low molecular weight substrates (lactic acid, acetic acid, ethanol hydrogen, etc.) to acetate as an incomplete oxidation product or carbon dioxide as a complete oxidation product to reduce sulfate [26]. Previous studies have shown that SRB plays an important role in the biogeochemical cycling of arsenic, and the sulfur and iron reduction processes mediated by SRB are considered to be an important mechanism affecting the transport and transformation of arsenic in anaerobic environments [27]. In addition to sulfate, sulfite, thiosulfate and elemental sulfur, SRB can also use nitrate, nitrite, iron and other compounds as electron acceptors.

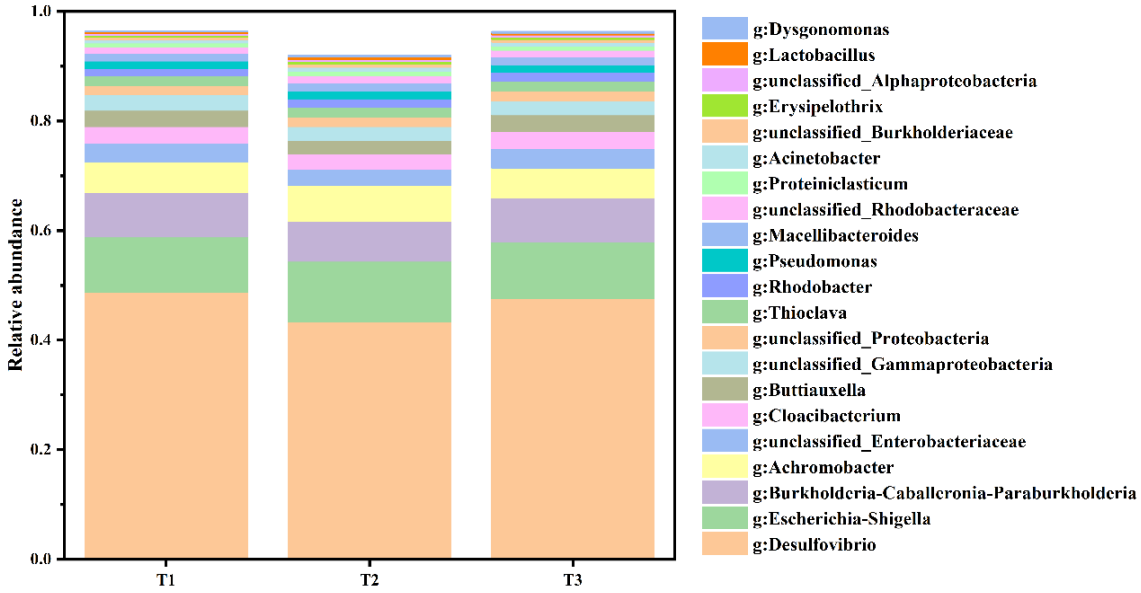


Figure 1. Relative abundance histogram of artificially enriched iron–sulfur-reducing microbial at the genus level.

3.2. Function of Iron–Sulfur-Reducing Microbial

The effect of iron–sulfur-reducing bacteria on the solubilization and differentiation of arsenic in soil was investigated under anaerobic incubation conditions, and the results are shown in Figure 2. As depicted in Figure 2a, the pH value of the leachate solution was hardly altered after the addition of iron–sulfur-reducing bacteria, similar to the control group. However, when plant organic matter was introduced, the pH value of the soil leachate increased, eventually reaching 7.39, with a difference of 0.31 compared to the initial period. This increase can be attributed to the anaerobic bacterial community dominated by Desulfovibrio, which becomes metabolically active in the presence of an organic carbon source. However, when plant organic matter was added, Desulfovibrio was able to oxidize organic acids produced by plant fermentation and transfer electrons to the sulfate in the soil, thus increasing alkalinity [28]. This reaction can be represented by the following reaction Equation (1):

$$C_2H_4O_2 + SO_4^{2-} \rightarrow 2HCO_3^- + H_2S \quad (1)$$

The concentration of dissolved As produced in the Bacterial + POM group was significantly higher than that in the other culture groups, as shown in Figure 2b. The concentration of As increased rapidly in the pre-culture period; by the end of the culture period, the concentration of As in the leachate had reached 4.76 mg/L, which was higher than that in the control group (3.24 mg/L). The above results suggested that the metabolism of iron–sulfur-reducing flora dominated by Desulfovibrio can promote the dissolution and release of arsenic from the soil under the supply of a sufficient organic carbon source. The first reasonable explanation is that HCO_3^- produced by the respiration of Desulfovibrio competed with arsenate and caused the desorption of arsenic. On the other hand, HS^- produced by the reduction of sulfate (via Desulfovibrio) created crucial conditions for the reduction of Fe(III), which could enhance the mobility of As by the reductive dissolution of iron oxides [29–31]. More importantly, the reduction products of sulfate could stimulate the reduction of As(V) to As(III) [32]. The reaction process is represented by the following Equations (2) and (3):

$$HS^- + 2FeOOH + 5H^+ \rightarrow 2Fe^{2+} + S^0 + H_2O \quad (2)$$

$$HS^- + HAsO_4^{2-} + H^+ \rightarrow S^0 + HAsO_3^{2-} + H_2O \quad (3)$$

Furthermore, it has been demonstrated that organic acids could promote the reductive solubilization of iron oxides, leading to the release of As bound to the iron oxides. The reaction process in the case of lactate can be expressed by the following Equation (4):

$$C_2H_4O_2 + 8FeOOH \rightarrow 8Fe^{2+} + 2HCO_3^- + 12H_2O \quad (4)$$

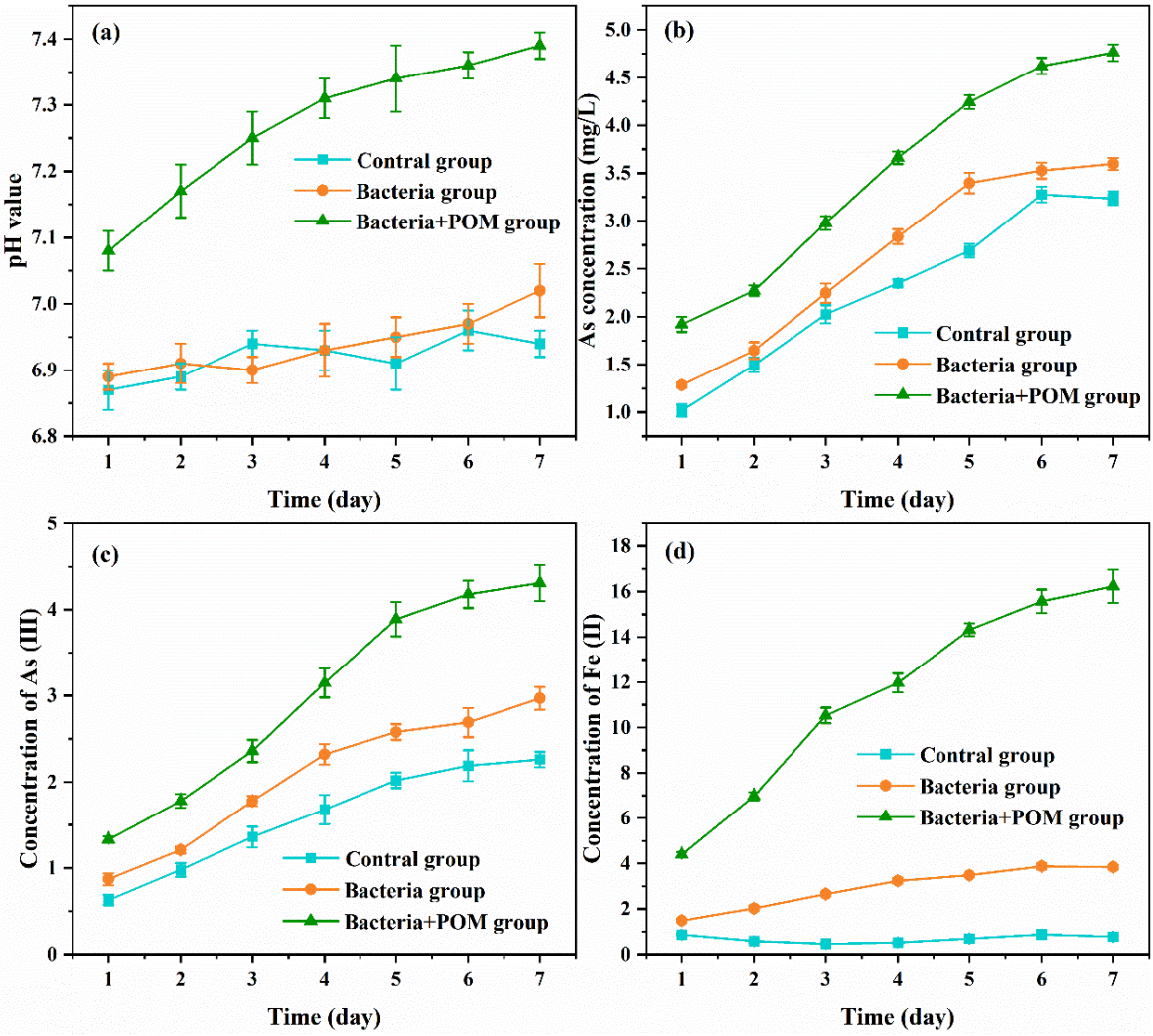


Figure 2. pH value of the supernatant (**a**), total As concentration of the supernatant (**b**), As(III) concentration of the supernatant (**c**), Fe(II) concentration of the supernatant (**d**).

In the presence of a specific dissolved organic matter, in addition to the reductive solubilization of As-containing iron oxides, As(V) could be reduced to As(III) by Desulfovibrio through an allochthonous reduction process. For instance, when lactate serves as the electron donor, the plausible direct reduction process of As(V) is described in Equation (5):

$$2HAsO_4^{2-} + CH_3CHOHCOO^- + H^+ \rightarrow 2H_2AsO_3^- + HCO_3^- + CH_3COO^- \quad (5)$$

In conclusion, iron–sulfur-reducing bacteria play a crucial role in facilitating the release and reduction of arsenic in contaminated soil. Firstly, they indirectly promote this process by controlling the reduction of iron oxides and sulfates present in the soil. Secondly, these bacteria directly contribute to the reduction of arsenic by transferring electrons to arsenic through the oxidation of organic matter as electron donors. Overall, the presence of iron–sulfur-reducing bacteria enhances the release and reduction of arsenic through

both indirect and direct mechanisms, making them crucial actors in the remediation of arsenic-contaminated soil.

3.3. Growth and Biomass of Pteris vittata after Bio-Reductive Enhancement

Plant biomass is a crucial indicator of plant growth in contaminated soil. The initial average height of *Pteris vittata* seedlings was measured at 12 ± 0.32 cm, and their biomass was determined to be 0.87 ± 0.07 g. As shown in Figure 3, *Pteris vittata* exhibited robust growth, with no observable toxic symptoms across all treatment groups. Furthermore, all treatment groups displayed a significant increase in plant height and biomass after four months of planting. Comparing the results to the control group (T1), it was found that the inoculation of iron–sulfur-reducing microbial flora and the application of plant organic matter (POM) (T5) significantly enhanced the plant height and biomass of *Pteris vittata*. Specifically, the plant height increased from 25.23 cm to 30.24 cm, representing an increment of 19.86 percentage points. The total biomass of *Pteris vittata* increased from 4.22 g to 4.92 g, indicating a 16.59 percentage point increase. Moreover, the biomass of roots and fronds increased by 36.11 percentage points and 9.87 percentage points, respectively. Additionally, a modest increase of 3.32 percentage points in *Pteris vittata* biomass was observed in the treatment group that was solely inoculated with iron–sulfur-reducing flora (T3). The introduction of plant organic matter further stimulated the growth of *Pteris vittata*. This was attributed not only to the provision of metabolic substrates for bacteria through the small-molecule organic acids present in plant organic matter [33], but also to the effective mobilization of soil nitrogen and available phosphorus, which promoted plant growth [34].

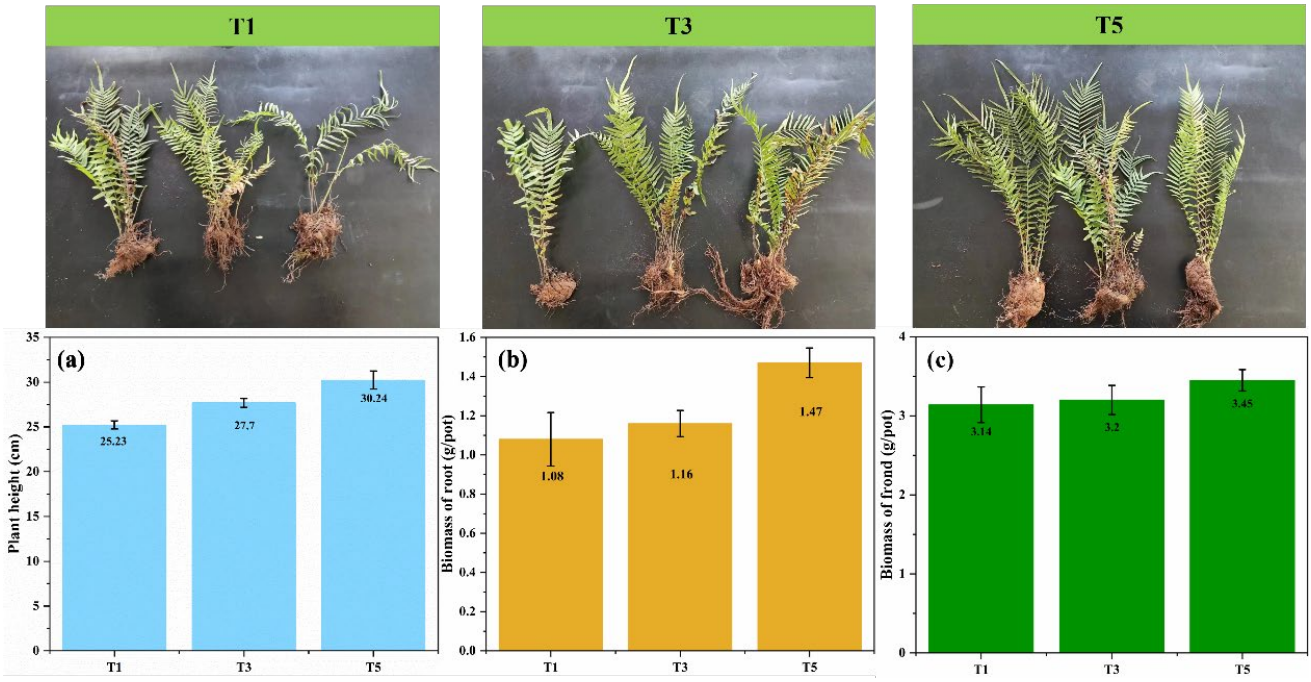


Figure 3. Plant height of *Pteris vittata* in each group (**a**), biomass of root (**b**), biomass of frond (**c**).

3.4. Extraction and Transport of Arsenic by the Pteris vittata

The ability of plants to absorb and enrich heavy metals is the key to the removal of heavy metals by phytoextraction. The enrichment content of arsenic in the roots and fronds of *Pteris vittata* is shown in Figure 4a. The content of arsenic in the fronds was significantly higher than that in the roots in all treatment groups. In the control group, arsenic content in the roots and fronds of *Pteris vittata* was 536.19 mg/kg and 1145.16 mg/kg, respectively. After inoculation with bacteria, the arsenic content in the roots and fronds

increased by 21.27 and 18.56 percentage points, respectively. Furthermore, when bacteria and organic matter were both introduced, the rise in arsenic content in the roots and fronds increased 47.60 and 37.46 percentage points. As depicted in Figure 4b, the accumulation of arsenic in the roots and fronds was 0.58 mg and 3.60 mg, respectively, in the control group. However, in the treatment group with the addition of bacteria and organic matter, the accumulation of arsenic increased to 1.41 mg in the roots and 6.43 mg the fronds. The overall arsenic accumulation per plant increased from 4.18 mg in the blank treatment to 7.84 mg in the biofortified treatment, resulting in an increased arsenic extraction rate of 87.56 percentage points.

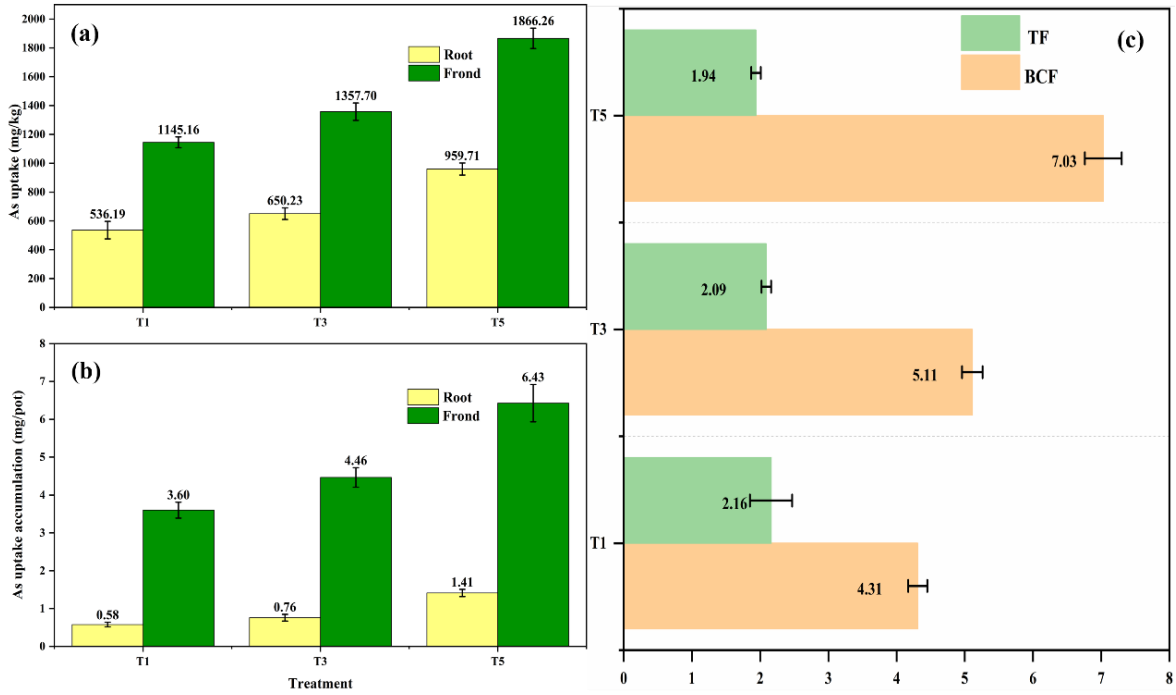


Figure 4. Arsenic absorption by *Pteris vittata*. (**a**) The concentration of arsenic in roots and fronds, (**b**) the cumulative amount of arsenic absorbed by roots and fronds, (**c**) arsenic BCF and TF of *Pteris vittata*.

The effectiveness of transferring arsenic contamination from soil to plant roots, as well as from roots to fronds, can be evaluated using the enrichment factor (bioaccumulation factor, BCF) and the translocation factor (TF). Figure 4c shows that the bioaccumulation factor (BF) for *Pteris vittata* ranged from 4.31 to 7.03 in all treatment groups, indicating that *Pteris vittata* is a typical arsenic hyperaccumulator and is highly efficient in removing arsenic from contaminated soil. The addition of bacteria and organic matter increased the bioaccumulation factor by 63.11 percentage points, indicating enhanced arsenic extraction by *Pteris vittata*. This may be attributed to changes in the soil's arsenic morphology under bacterial activity after inoculation, leading to the rapid uptake and accumulation of arsenic in the roots of *Pteris vittata*. On the contrary, the TF of the translocation coefficient of *Pteris vittata* decreased slightly, from 2.16 to 1.94, suggesting that arsenic transport from roots to stems has slowed down. Although the transfer of heavy arsenic roots to fronds contributes to plant tolerance, *Pteris vittata* may develop new detoxification mechanisms with the assistance of bacteria when exposed to high arsenic concentrations in the roots [34].

3.5. Speciation of Arsenic Uptake by Pteris vittata

To further understand the process of arsenic uptake by *Pteris vittata*, speciation examination of arsenic in *Pteris vittata* roots and fronds was performed, and the findings are presented in Figure 5 Arsenic in the roots and fronds of *Pteris vittata* was mainly present as As(III), with a higher proportion of As(III) in the fronds (83.87–87.29%) compared to the

roots (65.32–73.26%). Previous studies have proved that the valence state of arsenic did not affect the uptake rate in *Pteris vittata*. However, different valence states of arsenic were transferred at varying rates within *Pteris vittata*, with arsenic(III) being transferred from roots to fronds faster than arsenic(V). As a result, the concentration of arsenic(III) in the fronds was higher than that of arsenic(V). In the treatment group T5, where iron–sulfur-reducing bacteria and organic matter were introduced, the proportion of As(III) in both the roots and leaves of Pterocarpus indicus was significantly higher compared to the other two treatment groups. This increase can be attributed to the predominance of trivalent arsenic in anaerobic environments [35] and the metabolic activities of iron–sulfur-reducing bacteria, which enhance the solubilization of trivalent arsenic. As a result, *Pteris vittata* is able to take up trivalent arsenic more effectively. Previous studies have indicated that *Pteris vittata* roots absorb arsenate through the phosphate transport system, and the presence of phosphate competes with arsenate [36,37]. Phosphate inhibits arsenate uptake but not arsenite uptake and translocation, and arsenate is converted to arsenite by reductase in *Pteris vittata* roots and translocated into the fronds. Hence, the major form of arsenic in the fronds is arsenite, accounting for over 80% of the total, and the percentage of arsenite is higher in both the roots and fronds of the Bacterial + POM group compared to the control group. The mechanism of arsenite uptake by *Pteris vittata* differs from that of arsenate. Although the precise mechanism is not clear, several plausible explanations exist. One theory suggests that microbial-mediated oxidation of As(III) occurs on the surface of *Pteris vittata* roots, and the oxidized As is taken up by the roots through the phosphate transport system [38]. It then interacts with substances that compete with arsenate reductase (ACR) and glutathione (GSH), resulting in enzymatic or non-enzymatic reduction to arsenite [39]. And the reduction of As(V) to As(III) is a crucial step in the detoxification of *Pteris vittata* biomass. Another widely accepted viewpoint suggests that *Pteris vittata* can uptake As(III) via the aquaporins (AQPs) transport system [40]. It has also been observed that the AQP inhibitor $AgNO_3$ reduces the uptake of As(III) by *Pteris vittata* at a concentration of 0.01 mM, indicating that As(III) uptake may indeed be mediated by the AQP transport system [41], which differs from the transport system for As(V). Arsenite and phosphate do not compete during transport, making arsenite more readily translocated from roots to stems and leaves compared to arsenate.

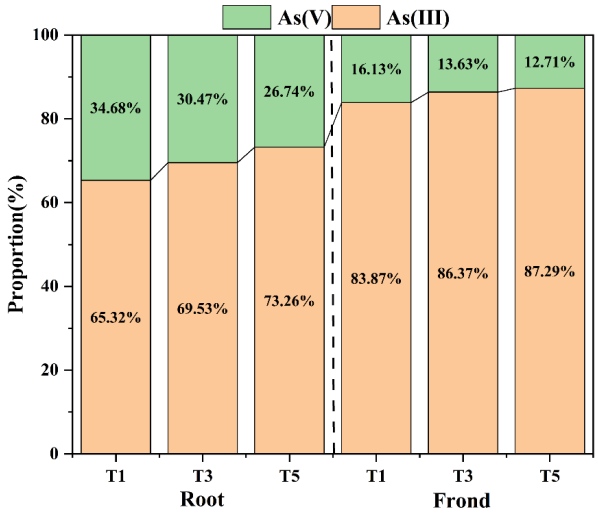


Figure 5. The speciation of arsenic in the roots and fronds of *Pteris vittata*.

In conclusion, the phytoremediation abilities of *Pteris vittata* in the uptake of soil arsenic can be attributed to three key roles it plays. Firstly, the roots of *Pteris vittata* provide a stable habitat for microorganisms and secrete organic acids that serve as nutrients for these microorganisms. Secondly, *Pteris vittata* employs distinct uptake mechanisms for both As(V) and As(III). Arsenate (As(V)) is taken up through the phosphate transport system,

where it competes with phosphates for uptake. Arsenite (As(III)), on the other hand, has its own independent uptake system known as the AQP transport system. Lastly, *Pteris vittata* is capable of enzymatic and non-enzymatic reduction of As(V) to As(III). This detoxification process not only ensures the plant's survival but also enhances the translocation of arsenic to the stems and leaves. These three roles collectively contribute to the efficient uptake and translocation of arsenic by *Pteris vittata*, making it an effective hyperaccumulator for the phytoremediation of arsenic-contaminated soil.

3.6. Changes in Soil Arsenic Content and Fractionation

To better evaluate the effectiveness of phytoremediation, changes in soil arsenic content were assessed. After *Pteris vittata* was growing for 4 months, significant changes in soil arsenic content were observed, as depicted in Figure 6a. The soil arsenic content decreased in all three treatment groups with *Pteris vittata*, with reductions of 11.95%, 14.86% and 19.62% in each group, respectively. The treatment group with the addition of iron–sulfur-reducing bacteria and organic fertilizer (T5) demonstrated the highest reduction in arsenic content. This indicates that the inclusion of iron–sulfur-reducing bacteria and organic fertilizer may enhance the uptake of arsenic by *Pteris vittata*, with organic fertilizers playing a particularly significant role.

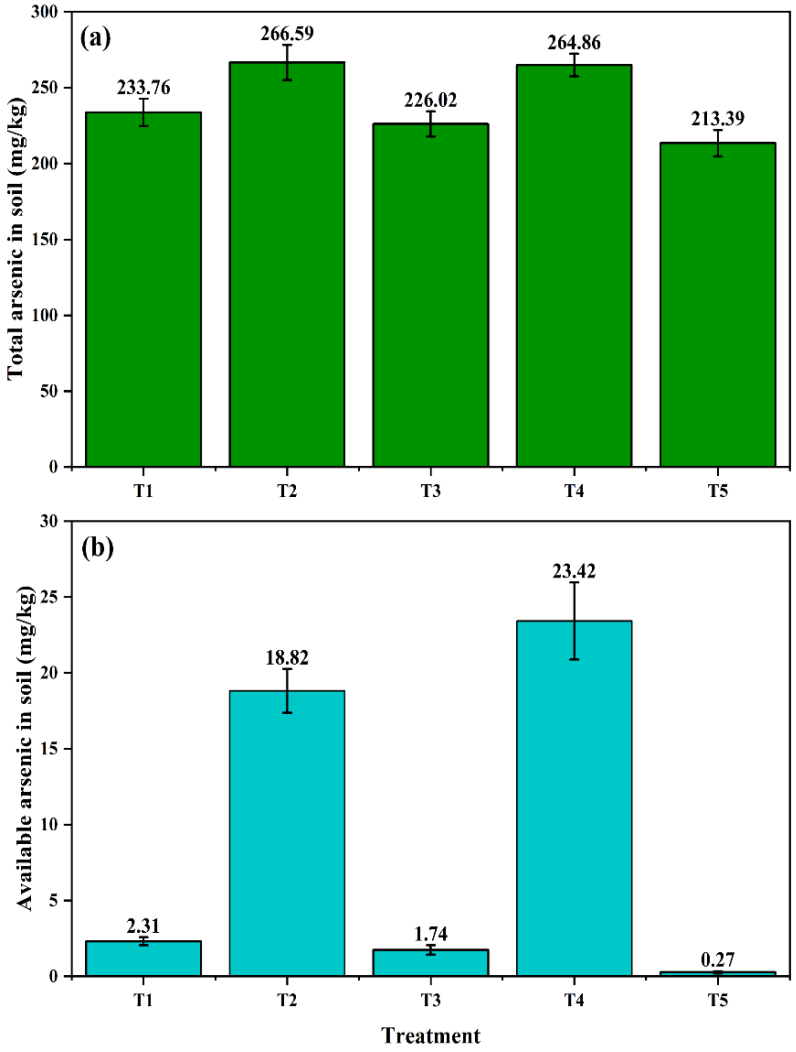


Figure 6. Total arsenic (**a**) and available arsenic (**b**) content of soil in each treatment group.

As illustrated in Figure 6b, in the control groups (T2, T4) without the planting of *Pteris vittata*, the soil arsenic content remained relatively stable, but there was a notable increase in available arsenic content. This can be attributed to the enhanced bacterial metabolic activity resulting from the presence of sufficient organic matter as a substrate. This, in turn, promoted the dissolution of acicular iron ore and hematite in the soil, leading to the release of arsenic and an increase in available arsenic content. The reduction rates of available arsenic in the three experimental groups after fern remediation were 85.88%, 89.41% and 98.36%, respectively, with the highest reduction rate observed in the bacteria + POM group. This suggests a synergistic relationship between the bacterial-induced transformation of arsenic species and the accumulation of arsenic by plants. Furthermore, the absorption of arsenic by *Pteris vittata* remained within its tolerance range, preventing arsenic leakage.

The BCR sequential extraction method was utilized to analyze the fractionation of arsenic in the soil following four months of remediation; the results are shown in Figure 7. The primary fraction of arsenic in the raw soil was the residual fraction (56.37%), followed by the reducible fraction (20.34%), the oxidizable fraction (13.44%) and the acid-soluble fraction (6.75%). Among these fractions, the acid-soluble fraction was identified as the most mobile and bioavailable form of arsenic. And the transport and transformation of arsenic in the reducible fraction were strongly influenced by chemical or microbially mediated redox processes of iron oxides in the natural environment [42]. Iron (hydro)oxides in soils, especially those with low crystallinity (hydrous iron ore, needle iron ore, etc.), provide the main adsorption sites for arsenic in soils [43]. And the reductive dissolution of iron (hydro) oxides is the main cause of arsenic release from soils or subsurface environments [44]. Consequently, in treatments T3, T4 and T5 inoculated with reducing bacteria, a significant decrease was observed in the reducible fraction of arsenic, indicating that the inoculation of reducing bacteria facilitated the reductive transformation of Fe(hydro) oxides in the soil, leading to the release of reducible arsenic. The reduction in the reducible fraction was more pronounced in treatment group T5, which can be attributed to the fact that the small molecular organic acids in the plant organic matter (POM) also contributed to the solubilization of iron oxides. A decrease in the reducible fraction of arsenic was also observed in the T1 and T3 treatment groups without POM application, as *Pteris vittata* itself secretes small amounts of organic acids from its roots during growth. The dissolution of iron (hydro) oxides resulted in a reduction in the sorption sites for arsenic, which was then released into the soil pore water as a free fraction and subsequently absorbed by the roots of *Pteris vittata*. The soils treated with Pteris vitt extraction (T1, T3, T5) exhibited a significant decrease in both the acid-soluble and reducible fractions, while an increase was observed in the oxidizable and residual fractions and a decrease in the migration and bioavailability of soil arsenic.

3.7. Transformation of Soil Element Valance

XPS was used to analyze the changes in the elemental chemical states of As, S and Fe in the soil before and after phytoremediation in order to further clarify the mechanism of functioning of iron–sulfur-reducing bacteria. The XPS spectra of As 3d, Fe 2p and S 2p are illustrated in Figure 8.

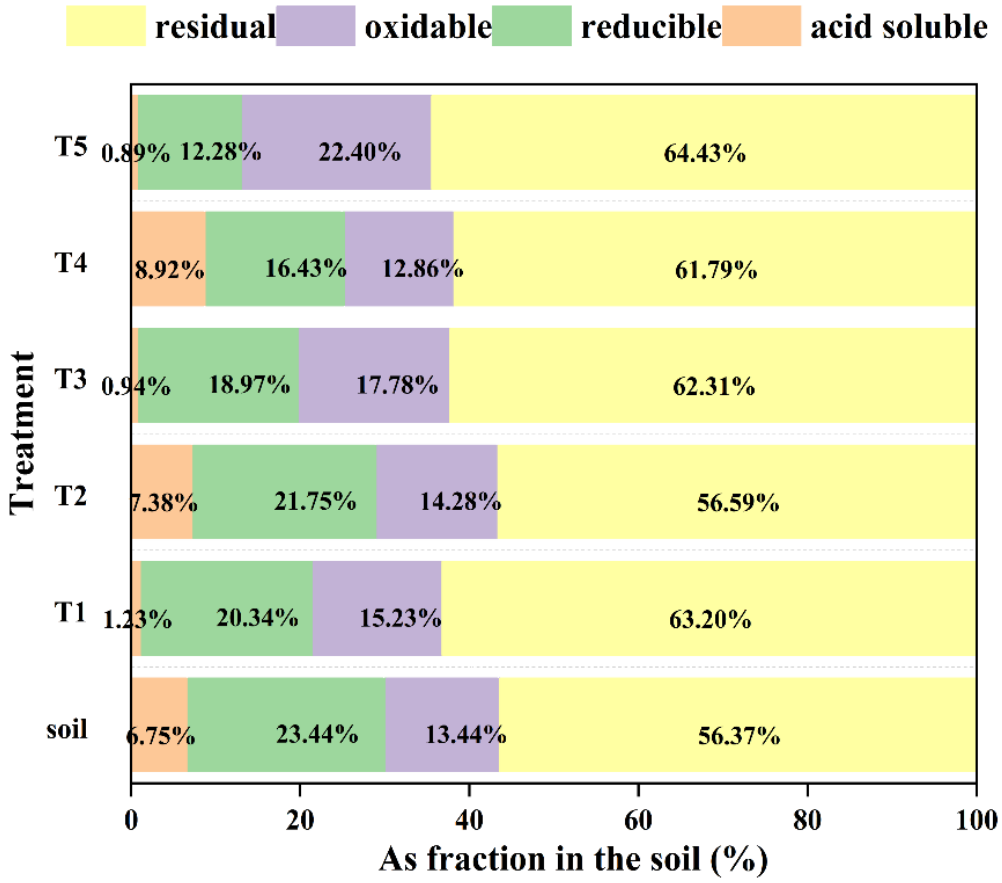


Figure 7. Fractionation of arsenic in soil after phytoremediation.

The peaks at 44.1 eV and 44.8 eV corresponded to As(III) 3d3/2 and 3d5/2, respectively, while the peaks at 46 eV, 46.7 eV, 49.3 eV and 50 eV were assigned to As(V) [45]. After phytoremediation, the proportion of As(III) in the soil decreased from 37.68% to 34.28%. This could be attributed to the higher mobility of As(III), while As(V) readily resorbs on the soil surface. Consequently, the higher levels of As(V) detected on the soil surface by XPS may have influenced this result. Regarding sulfur, the peaks at 164.2 eV and 165.8 eV corresponded to S(II) 2p1/2 and 2p3/2 of FeS, respectively, while the peaks at 167.3 eV and 168.5 eV were assigned to S(II) 2p1/2 and 2p3/2 of FeS2. The peaks at 170.2 eV and 171.4 eV were attributed to S(VI) 2p1/2 and 2p3/2 of sulfate. As watering during the experiment did not result in the loss of sulfate, the decrease in sulfate content from 48.56% to 30.27% after remediation suggests that the metabolic activity of iron–sulfur-reducing bacteria was responsible for the reduction in sulfate levels in the soil. In terms of iron, the peaks at 725.0 eV and 711.8 eV corresponded to Fe(III) 2p1/2 and 2p3/2 of Fe_2O_3 or FeOOH [46,47], respectively. The peaks at 727.0 eV, 723.5 eV, 713.8 eV and 710.2 eV were attributed to Fe(II) 2p1/2 and 2p3/2, while the peak at 719.0 eV represented the Fe(III) satellite. The proportion of Fe(III) decreased from 42.27% to 40.14% after phytoremediation, indicating that the iron–sulfur-reducing bacteria and plant organic matter (POM) contributed to the reductive dissolution of iron oxides.

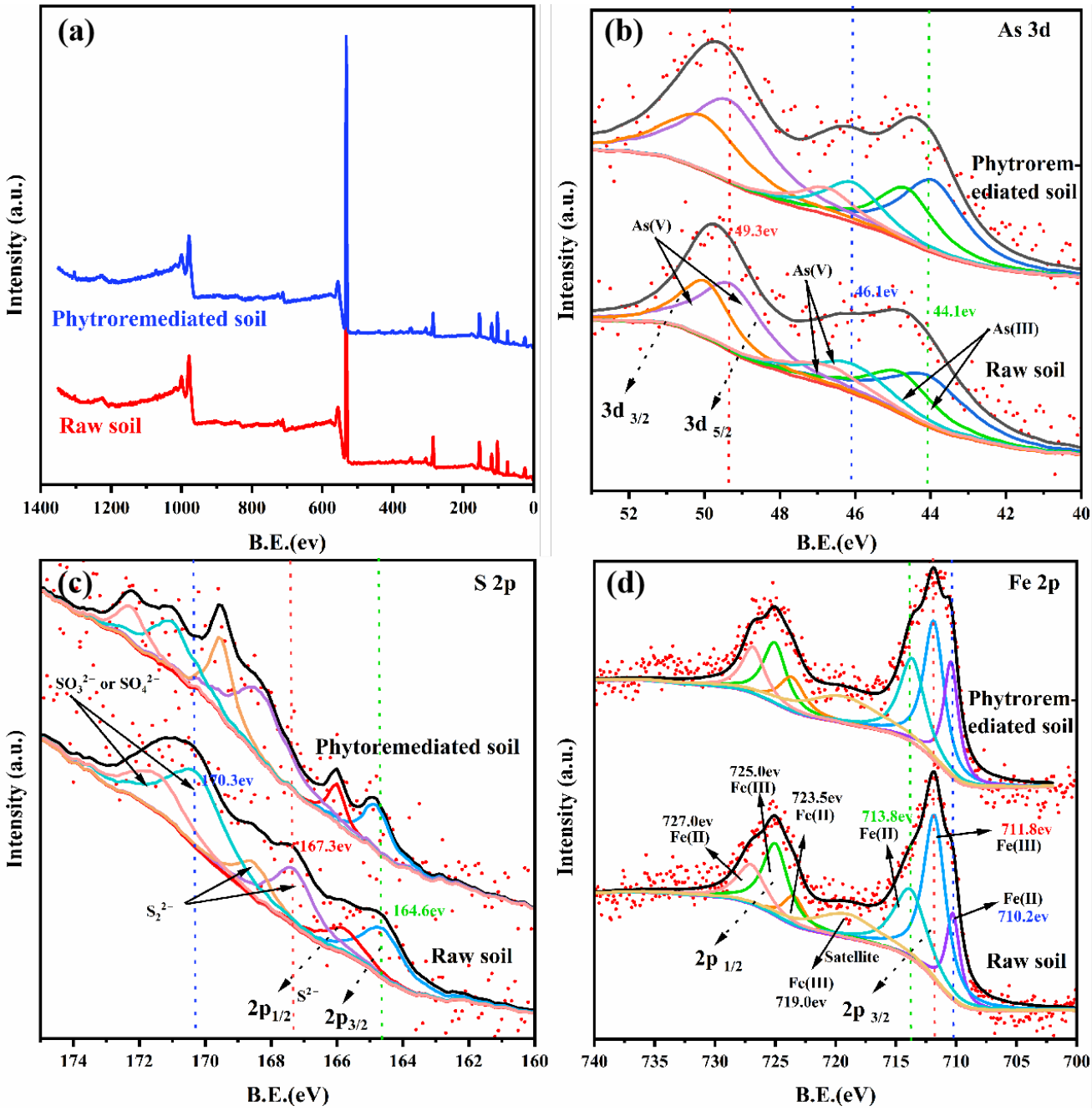


Figure 8. Full spectrum of XPS scans of soils (**a**), XPS spectra of As3d (**b**), S 2p (**c**), Fe 2p (**d**) of raw arsenic-contaminated soil and soil treated after phytoremediation (T5 group).

3.8. Effects of Iron-Sulfur-Reducing Flora on Soil Microbial Community

The bacterial composition of the raw soil and rhizosphere soil consisted primarily of 15 phyla, including Proteobacteria, Actinobacteria, Gemmatimonadetes, Deinococcus-Thermus, Chloroflexi, Acidobacteria, Euryarchaeota, Bacteroidetes, Planctomycetes and others. Proteobacteria and Actinobacteria were the dominant phyla, accounting for a cumulative abundance ranging from 65.28% to 75.86% (Figure 9a). In terms of bacterial genus, Pseudomonas and Burkholderia were the most prevalent in the community (Figure 9b). Notably, the relative abundance of Desulfovibrio in the rhizosphere soil of *Pteris vittata* increased significantly with the application of iron–sulfur-reducing bacteria and plant organic matter, compared to the raw soil. This suggests that Desulfovibrio is well adapted to the soil environment and exhibits steady proliferation.

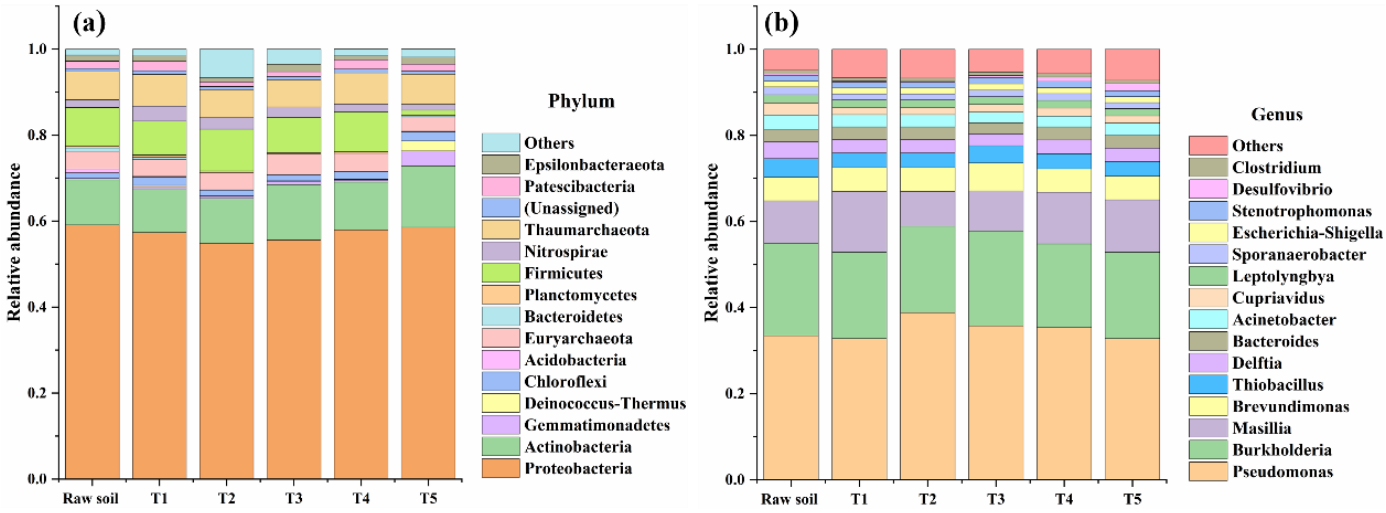


Figure 9. Effects of iron–sulfur-reducing bacteria on the phylum (**a**) and genus (**b**) composition of the bacterial community in rhizosphere soil cultivating *P. vittata*.

3.9. Mechanism of Bio-Reductive Enhanced Phytoremediation

The toxicity and bioavailability of arsenic in soil are determined by its chemical fractionation and state, with the mobility of arsenic closely linked to the geochemical cycling processes of sulfur and iron. Heavy metals in soil can be categorized into acid-soluble, reducible, oxidizable and residual fractions. The acid-soluble fraction exhibits greater mobility, while the reducible and oxidizable fractions are relatively stable but prone to transformation through redox reactions [48,49]. To address this, artificially cultured iron–sulfur-reducing microorganisms were utilized to promote the reduction of sulfate and trivalent iron, consequently reducing the reducible fraction in soil. This process leads to the release of arsenic bound to iron oxides or adsorbed on their surfaces, allowing it to enter the pore water as free arsenic fractions that can be absorbed by *Pteris vittata* (Figure 10).

In anaerobic environments, organic carbon sources serve as energy sources for heterotrophic bacteria. Small molecules, such as lactic acid and acetic acid produced during the fermentation of plant straw, are oxidized and decomposed by iron–sulfur-reducing bacteria. The resulting electrons are transferred to electron acceptors such as sulfate and Fe(III). Arsenic(V) can also serve as a direct electron acceptor or be reduced by S^{2-} and Fe^{2+}. Additionally, the organic matter derived from fermented plants provides nutrients for the growth of *Pteris vittata*, promoting increased biomass [50]. The uptake of pentavalent arsenic by *Pteris vittata* proceeds through the phosphate system, and thus there is competition between arsenate and phosphate, but the uptake of arsenite proceeds through an independent system. As(III) content can be increased through direct reduction by organic acids as well as indirect reduction by S^{2-} and Fe^{2+} produced by iron–sulfur-reducing bacteria's metabolism. This increase in As(III) content facilitates the uptake and translocation of arsenic by the root system of *Pteris vittata*.

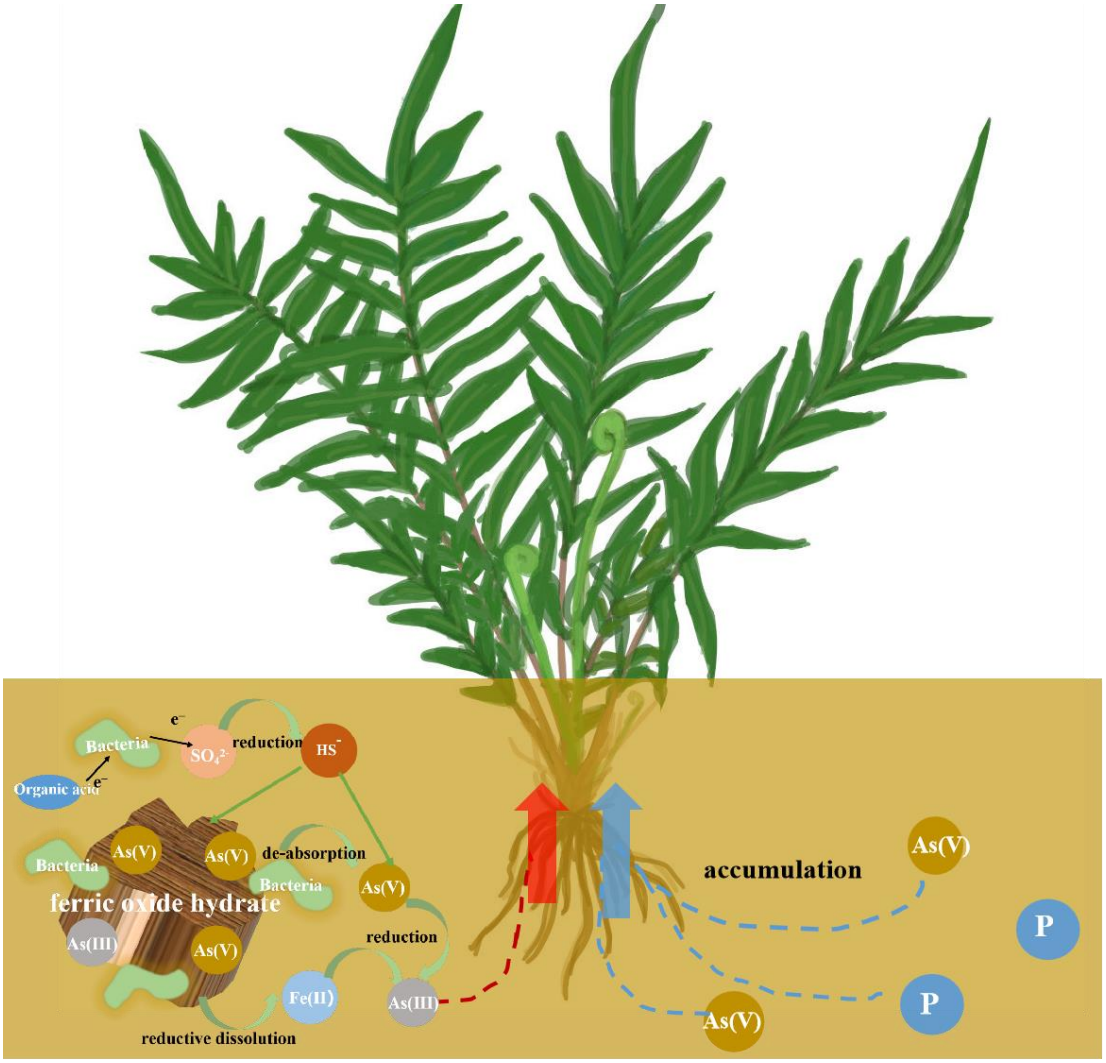


Figure 10. Mechanism of phytoremediation of *Pteris vittata* enhanced by bio-reduction.

4. Conclusions

In this study, the functional iron–sulfur-reducing bacterial flora in soil was artificially enriched, which mainly consisted of Desulfovibrio, Escherichia-Shigella, Paraburholderia and Achromobater. The functional bacteria were used in the study of phytoremediation of arsenic-containing soil by *Pteris vittata*. Instead of destroying the structure of the soil microbial community, the inoculation of the bacterial solution showed good integration, which was mainly reflected in the increase in the relative abundance of Desulfovibrio.

After a four-month phytoremediation period, the results revealed that the iron–sulfur-reducing bacteria effectively catalyzed the reductive dissolution of sulfate and iron oxides in the soil, promoting the transformation of arsenic in the reducible fraction to bioavailable arsenic. This transformation relied on the presence of organic matter to support the metabolic activities of the iron–sulfur-reducing bacteria. Consequently, the addition of straw-fermented organic fertilizer facilitated the above reactions and also provided essential nutrients for *Pteris vittata*, resulting in increased biomass.

The metabolic activities of iron–sulfur-reducing bacteria and the small-molecule organic acids present in the organic fertilizers contributed to an increase in arsenite release. *Pteris vittata* exhibited distinct uptake mechanisms for arsenate and arsenite, with arsenate being taken up through the phosphate system and competing with phosphates, while arsenite utilized an independent system. As a result, the accumulation and transport of

arsenic in *Pteris vittata* were facilitated by the elevated concentrations of bioavailable arsenic and arsenite.

Ultimately, with the assistance of iron–sulfur-reducing bacteria and organic fertilizers, the biomass of *Pteris vittata* increased by 16.59%, and arsenic accumulation was elevated by 87.56%. Overall, the synergy between bio-reduction processes mediated by iron–sulfur-reducing bacteria and the specific uptake mechanisms of *Pteris vittata* plays a crucial role in the efficient removal and remediation of arsenic from contaminated soil. These findings indicate that the utilization of iron–sulfur-reducing bacteria in assisting the phytoremediation of *Pteris vittata* is a highly effective strategy with promising application prospects.

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