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Phytoremediation of Tungsten Tailings under Conditions of Adding Clean Soil: Microbiological Research by Metagenomic Analysis

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Abstract: Vegetation coverage of metal tailings is an important method for environmental governance. Colonization of plants on some nutrient-poor tailings is difficult. Therefore, the addition of clean soil (CSA) is needed to support plant growth. However, the promotion of plant growth by CSA has been widely reported, and there is a lack of reports on the effects of CSA on soil microbial communities and nutrient cycling-related genes. In this study, using ryegrass as the selected plant, the phytoremediation of tungsten tailings was conducted under conditions of CSA. The research focused on investigating the variation in the microbial community's structure and elucidating variations in the metabolic pathways and relative abundance of nutrient cycling genes. The results suggest that CSA and planting ryegrass increased the microbial richness in tailings. CSA had a negative impact on the microbial community's evenness (Shannon index) and richness (Simpson index). In all treatments, the relative abundance of *Pseudomonadota* ranged from 64.4% to 75.2% and dominated the microbial community. High levels of CSA (T3) reduced the relative abundance of *Pseudomonadota* by 10–13%, and a higher relative abundance of *Ascomycota* was observed after planting ryegrass. At the genus level, the growth of ryegrass benefitted from a decrease in the abundance of *Pseudomonas*, *Phenobacterum*, and *Sphingobium* after CSA. Cultivation of ryegrass increased the relative abundance of the nitrogen-fixing bacterium *Bradyrhizobium* (0.9%), which is beneficial for the sustainability of soil remediation in tailings. Metabolism was the primary activity process of microorganisms in tailing soil, with a relative abundance of 71.3% to 72.7%. Generally, the changes in the microbial community's composition indicated that CSA and cultivation of ryegrass were beneficial for tailings. Still, the negative effects of CSA on microbial evenness (Shannon index) and richness (Simpson index) need attention.

Keywords: phytoremediation of tailings; addition of clean soil; microbial metabolic activity; microbial community structure; nutrient cycling

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1. Introduction

Tungsten, a crucial metallic resource, finds widespread applications in various facets of life and technology. Consequently, numerous countries globally exhibit a pressing demand for tungsten. China, boasting the largest tungsten reserves, contributes to over 60% of the global account and is the leading producer of tungsten concentrates worldwide [1]. With several decades of history, tungsten mining in China has accumulated substantial tailings due to prolonged extraction activities [2]. Managing solid waste in the mining industry poses a formidable environmental challenge. The adverse environmental impacts of mine tailings primarily manifest in two aspects: firstly, the heavy metals present in the tailings undergo leaching into environmental systems such as the soil and groundwater under

the influence of rainfall; secondly, extensive storage of tailings encroaches upon valuable land resources.

The transformation of tailings into productive land is not an immediate necessity. Accumulated tailings possess a loose matrix, making them prone to secondary disasters. Numerous studies have been conducted to address the remediation of tailings. Liu et al. [3] investigated the feasibility of soilless cultivation for the restoration of gold tailings. The results indicated a decrease in the tailings' pH (by 0.67 to 1.11 units), a reduction in the heavy metal content (by 29.8% to 44.0%), and an increase in the nutrient content (by 50.3% to 169.5%) after plant-based restoration. Wu et al. [4] used fast-growing tree species for the ecological restoration of manganese tailings. Applying organic microbial fertilizer yielded superior restoration results compared with the control group. After the application of organic microbial fertilizer, plant biomass, the accumulation of Mn, and plant species increased by 21.8, 1.6, and 2.4 times, respectively. The organic microbial fertilizer improved the community's structure and function, enhancing biodiversity in the study area. Jiang et al. [5] reported that CSA on lead–zinc mine tailings promoted the reconstruction of vegetation, increasing vegetation coverage by over 85%. Compared with the control group, the Shannon diversity index of bacteria increased, and the abundance of genes related to carbon and nitrogen cycling increased by 17% and 43%, respectively.

For tailings with poor nutrients for plant growth, the addition of clean soil (CSA) has been used to improve the physicochemical properties of tailings and promote plant growth. Sajeevee et al. [6] demonstrated the restoration of iron tailings through technical application of soil and planting ryegrass. Fang et al. [7] reported that CSA reduced the content of antioxidant enzymes in plants on lead–zinc tailings, alleviating the heavy metals' toxicity and increasing plant biomass. Bigot et al. [8] pointed out that CSA combined with phytoremediation was an effective measure for the restoration of tailings in a tailing reservoir. A certain amount of additional clean soil and plant vegetation were important measures to realize the ecological closure of tailings in the reservoir area. CSA allows for the injection of microorganisms into the tailings, with specific rhizosphere microorganisms promoting the development of a microbial community structure. Lin et al. [9] reported that the inoculation of rhizosphere microorganisms reshaped the microbial community's structure in tailings. CSA can directly enhance the suitability of tailings by increasing the nutrient content, adsorbing dissolved heavy metals, and maintaining the water content [10].

Overall, CSA has emerged as a promising method to facilitate the phytoremediation of tailings because it can increase plant biomass. However, there are few studies on the changes in microbial community's structure and microbial genes' function caused by phytoremediation under conditions of CSA. Ryegrass is a fast-growing plant that can quickly establish vegetation on tailings. Ryegrass has a well-developed root system and may potentially improve the loose physical structure of tailings [11]. Previous studies have reported that ryegrass can serve as a pioneer plant for vegetation on metal tailings after CSA [6,12]. Above all, this study planted ryegrass on tungsten tailings with three levels of CSA. The scientific questions addressed include: (1) investigating the variation in the microbial community's structure under different treatment conditions; and (2) elucidating variation in the relative abundance of metabolic and nutrient cycling genes.

2. Materials and Methods

2.1. Materials

The tailings used in this study were sourced from Ganzhou City, Jiangxi Province, China. The primary heavy metal pollutants in the tailings were Cd, W, and Pb, with 9.5, 642.0, and 136.8 mg/kg, respectively, and a pH of 7.65. Further details about the tailings can be found in our previous report [13]. The soil used for CSA was obtained from uncontaminated forested areas around the mine, addressing the limitations posed by the source and transportation costs of soil. The properties of the clean soil and the tailings are provided in Table S1. Under natural succession, tailings obtained from selected tailing ponds contain a small amount of nutrients but far lower than those in clean soil.

Ryegrass seeds for plant restoration were purchased from Shouhe Seed Industry (https://mall.jd.com/index-706749.html (accessed on 22 May 2024)).

2.2. Experimental Design

The tungsten tailings and soil were mixed in different proportions to obtain the tailing soils. Three treatment levels of CSA were designed: T1 (0% soil + 100% tailings), T2 (20% soil + 80% tailings), and T3 (50% soil + 50% tailings). The resulting tailing soils were placed in pots with a diameter of 12.5 cm, with each pot containing 500 g of tailing soils. Two sets of pots were prepared: one set where 20 ryegrass seeds were sown in each pot, serving as the planting (P) group, and another set without the cultivation of ryegrass, serving as the control (C) group. Therefore, there were 2 groups (C and P) and 6 treatments (CT1, CT2, CT3, PT1, PT2, PT3), each with three replicates. After germination of the seeds, the plants were allowed to grow for an additional 2 months in a greenhouse before harvesting. The greenhouse temperature was maintained at 25 °C, with 16 h/per day of lighting, and the intensity of the light was set at 120 μmol/(m^2/s). All pots were incubated at a selected moisture content by weight for 60 days. Specifically, deionized water was added every 3 days to maintain a 20% moisture content.

2.3. Experimental Methods and Data Analysis

2.3.1. Sample Collection and Analysis

After harvesting of the ryegrass, the root and shoot biomass of ryegrass were determined. The basic physicochemical properties of the tailing soils, including pH, organic matter (OM), and available nitrogen, phosphorus, and potassium content, were measured. The total and bioavailable concentrations of Cd, W, and Pb in the tailing soils were also determined. Fresh soil samples were collected for metagenomic analysis. The pH of tailing soils was measured using a pH meter with a liquid-to-solid (L/S) ratio of 2.5. The organic matter (OM) in the tailing soils was determined using the potassium dichromate–sulfuric acid method with heating in an oil-bath. The bioavailability of heavy metals (A_Cd, A_W, and A_Pb) was determined using the EDTA-2Na extraction method, with the concentrations of Cd, W, and Pb in the leachate measured by ICP-MS [14]. Microwave digestion was used to digest the tailings, and the concentrations of Cd, W, and Pb in the digested solution were determined. Available nitrogen (AN) was measured using the alkali diffusion method, available phosphorus (AP) was determined using the molybdenum antimony colorimetric method, and the content of available potassium (AK) was determined using flame atomic absorption spectrometry [15,16].

2.3.2. Metagenomic Analysis of Tailing Soils

DNA was extracted from the tailing soils using the CTAB method [17]. The extracted DNA's concentration, integrity, and purity were assessed using the Agilent 2100 system. The NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA) was used for construction of the library. Qualified DNA samples were randomly fragmented into fragments of approximately 350 bp in length using a Covaris ultrasonic breaker. The DNA fragments underwent end repair, addition of polyA tails, sequencing of adapter ligations, purification, and PCR amplification to complete the entire process of library preparation. Finally, the PCR products were purified using the AMPure XP system, the insert size of the library was detected using Agilent 2100, and the library's concentration was quantified using real-time PCR. Metagenomic sequencing of the bacterial communities in the tailing soil samples was performed using the Illumina high-throughput sequencing platform.

2.3.3. Data Analysis

SPSS 20.0 was used for statistical analysis of the data. The KEGG database was used for KEGG annotation, and the genes were classified into six metabolic pathways, including cellular processes, processing environmental information, processing genetic information,

human diseases, metabolism, and organismal systems, according to the KEGG PATHWAY database used to predict the microbial community's functions.

2.4. Statistical Analysis

Microsoft Excel 2016 was used to analyze the data, and then the related charts were drawn by OriginPro (2019); some charts were drawn by R4.0.0. All values are shown as the mean ± standard deviation, and all treatments were in triplicate. SPSS 20.0 was used to perform one-way analysis of variance (ANOVA), and all statistical tests were considered significant at p values < 0.05.

3. Results and Discussion

3.1. Overview of Variation in Agricultural Indicators

This study aimed to explore the impact of remediation procedures on microorganisms. The agricultural indicators (growth of ryegrass, physical and chemical properties of the tailings) are not discussed and have only been briefly reported. Briefly, adding clean soil promoted the growth of ryegrass and increased its biomass (Table S2). As reported in many studies, this is attributed to the higher nutrient content in clean soil (Table S1). The addition of clean soil changed the pH of tailings, and the bioavailability and total amount of heavy metals. Ryegrass grows on a mixture of tailings and soil, which is a process of consuming nutrients (AN, AP, and AK). Additionally, pH and OM changed under the rhizosphere's action. More details of the results can be found in the Supplementary Materials.

3.2. Analysis of Microbial Diversity

The microbial diversity of the six samples was analyzed using α-diversity measures, as shown in Table 1. The Chao 1 index represents species richness, and both CSA and planting ryegrass increased the Chao 1 index. The Shannon index and Simpson index decreased with an increasing proportion of CSA, indicating that CSA had a negative impact on the evenness and richness of the microbial community. Planting ryegrass increased the Shannon index but had a minor impact on the Simpson index, indicating an increase in species diversity due to planting ryegrass. The β-diversity analysis through a bar coordinate plot revealed the impact of CSA and planting ryegrass on the structure of the microbial community in tailings (Figure 1). The microbial communities in both the no-planting treatment (C) and the planting treatment (P) exhibited similar changes with an increasing amount of CSA. In summary, the increase in the proportion of CSA gradually shifted the distribution of microbial communities towards the bottom-right corner. Under the same proportion of CSA, the microbial communities in the tailings aggregated towards the upper-right side of the coordinate axis after planting ryegrass. The microbial communities in the PT2 and PT3 treatments were closely clustered in the coordinate system and significantly separated from CT1, indicating that simultaneous treatment with CSA and planting ryegrass could significantly modify the microbial community in the tailings. CSA directly modified the physicochemical properties of the tailings (e.g., available nitrogen, available phosphorus, available potassium, organic matter, etc.). At the same time, planting ryegrass indirectly affected the physicochemical properties of the tailings through plant processes (e.g., absorption of inorganic salts and heavy metals, secretion of small-molecule organic acids, promotion of bioweathering of the tailings).

Table 1. The microbial α diversity index in tailing soils.

Samples	Chao	Shannon	Simpson
CT1	1503.16	5.56	0.93
CT2	1649.05	5.47	0.91
CT3	1665.66	5.35	0.89
PT1	1661.25	5.74	0.93
PT2	1724.10	5.64	0.91
PT3	1778.38	5.56	0.90

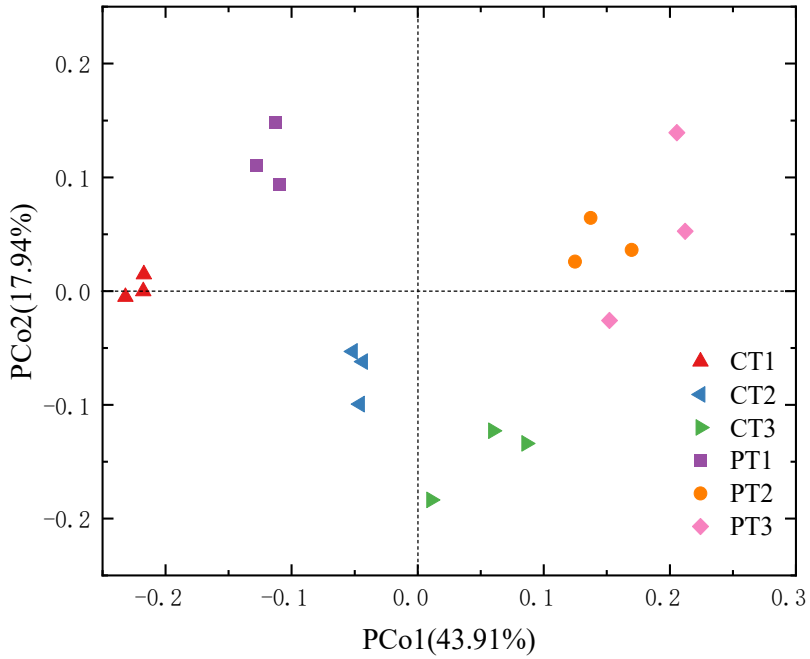


Figure 1. PCoA analysis of the six samples based on the microbial community's composition.

3.3. Variation in the Microbial Composition and Structure

After adding clean soil and planting ryegrass, the structure of the microbial community in the tailings at the phylum level was analyzed (Figure 2). The relative abundance of microorganisms varied with the treatments, and *Pseudomonadota* dominated the microbial community. In all six treatments, the relative abundance of *Pseudomonadota* ranged from 64.4% to 75.2%. *Pseudomonadota* is known for its strong tolerance of heavy metals and is often dominant in environments contaminated with heavy metals [18]. Yan et al. reported that the relative abundance of *Pseudomonadota* was significantly positively correlated with the concentration of heavy metals, indicating that it had significant adaptability to heavy metal environments [19]. The relative abundance of *Pseudomonadota* decreased with the addition of clean soil, which was attributed to the reduction in the total content of heavy metals. In CT3, the relative abundance of *Pseudomonadota* decreased by 13.0% compared with CT1. In contrast, in the ryegrass treatment (PT3 vs. PT1), the decrease was only 10%, possibly due to weathering of the tailings or activation of heavy metals induced by the plants' growth. Previous studies have shown that root exudates from plants can promote the migration of heavy metals in tailings [12]. *Actinomycetota*, ranking second in bacterial abundance after Proteobacteria, had a relative abundance ranging from 2.6% to 5.9%. The relative abundance of *Actinomycetota* increased with the proportion of SC, and the plants had a promoting effect. The addition of clean soil directly increased the organic matter content in the tailings. *Actinomycetota* can adapt well to environments with high levels of heavy metals in tailings, as they harbor metal resistance genes with a high relative abundance [20]. *Actinomycetota* plays a crucial role in the cycling of substances such as organic matter [21], and it can settle in plant roots, promoting the dissolution of insoluble phosphorus in minerals and nitrogen fixation, thereby promoting plant growth [22]. It also explains the promoting effect of planting ryegrass on the relative abundance of *Actinomycetota*. *Ascomycota* is an essential participant in the decomposition of plant residues and generally has a higher relative abundance in soils with vigorous plant root growth and good nutrient conditions, especially those with a high organic matter content. Rasamiravaka et al. [23] reported a significant positive correlation between *Ascomycota* and nutrients. Therefore, in this study, tailings treated with ryegrass had a higher relative abundance of Ascomycota than those without ryegrass (1.1% vs. 0.5%).

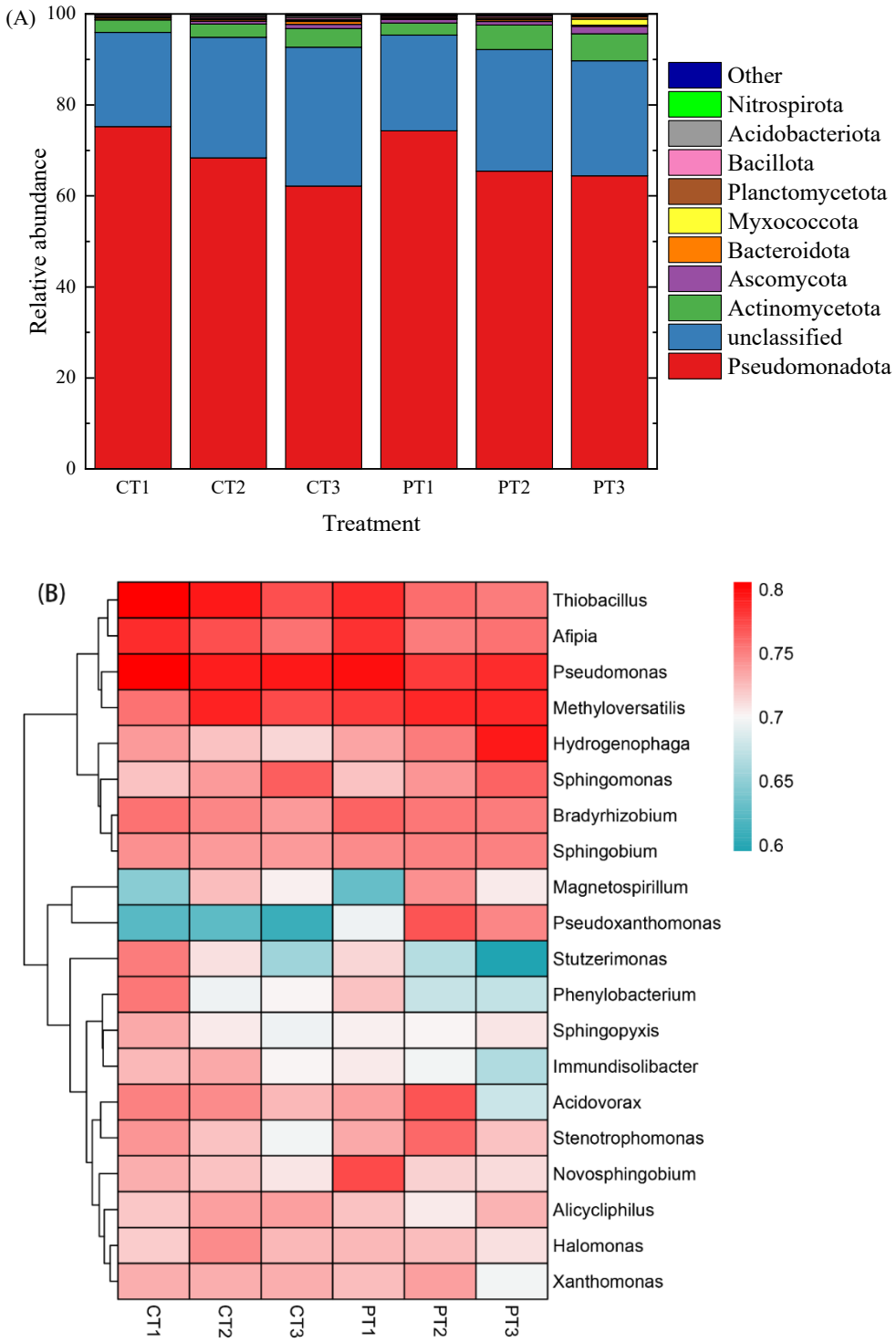


Figure 2. (**A**) Relative abundance of the most abundant bacteria at phylum level. (**B**) Heat map of the 20 most abundant genera. The corresponding value of the thermal map for each row of genera is the relative abundance after log10 conversion.

To further investigate changes in the microbial community's structure at the genus level during the restoration period, Figure 2 shows the 20 most abundant genera. The relative abundance of *Pseudomonas* ranged from 6.0% to 11.8%, and CSA reduced its relative abundance from 10.5–11.8% to 6.0–9.0%. Additionally, the planting treatment (8.1%) had a lower relative abundance of *Pseudomonas* than the no-planting treatment (10.4%). *Pseudomonas* is a conditionally pathogenic bacterium [24], and proteins rich in metallothioneins

and cysteine are its primary means of resisting heavy metals in environments such as soil [25]. Reducing the abundance of *Pseudomonas* is beneficial for alleviating the harm of heavy metals to plants in tailings. Further, the growth of plant roots promoted a decrease in the abundance of pathogenic bacteria, a sustainable process that is essential for promoting the restoration of plants in tailings. The relative abundance of *Methyloversatilis* increased with the proportion of CSA and had a higher relative abundance in the ryegrass treatment. *Methyloversatilis* is a methylotrophic microorganism with strong metabolic capabilities that is capable of utilizing methanol and methylamine, as well as many organic acids, alcohols, and aromatic compounds [26]. The increase in the organic matter content in tailings due to CSA and planting ryegrass provides a carbon source for its growth and reproduction. Additionally, Li et al. [27] first reported *Methyloversatilis* to be a genus with capabilities for the oxidation of sulfur. The tailings used in this study were from tungsten mines associated with sulfide minerals, with a sulfur content of 0.2%, providing a material basis for *Methyloversatilis*. Cluster analysis divided the 20 genera into two clusters, where *Methyloversatilis*, *Sphingomonas*, and *Hydrogenophaga* were the main degrading genera and exhibited the same changing trend. The relative abundance of aerobic microorganisms increased with the increase in organic matter in the tailings, directly leading to a decrease in the relative abundance of autotrophic organisms, and this also explained why *Methyloversatilis* and Thiobacillus, both with capacity for the oxidation of sulfur, showed different changing trends. Moreover, the decrease in the sulfur component due to the reduction of the proportion of tailings also explained the decrease in the relative abundance of *Thiobacillus*. *Stutzerimonas*, *Phenylobacterium*, *Sphingobium*, and *Immuudisolibacter* are low-abundance degrading genera, and their relative abundance decreased with CSA and planting ryegrass. Studies have shown that *Stutzerimonas*, *Phenylobacterium*, *Sphingobium*, and *Immuudisolibacter* play a role in degrading difficult-to-degrade organic substances (such as hexachlorohexane, petroleum hydrocarbons, and aromatic compounds) [28–30]. In CT1, the relative abundance of these genera was the highest, possibly due to the residual agents in the process of beneficiation. These residual agents were diluted or consumed with the addition of CSA and planting ryegrass, decreasing the abundance of degrading genera that use these substances as carbon sources. Furthermore, *Phenylobacterium* and *Sphingobium* also belong to the pathogenic bacteria, and the decrease in their abundance represents an improvement in the adaptability of tailings, which is conducive to planting ryegrass. *Bradyrhizobium* is an important genus of nitrogen-fixing bacteria that plays a crucial role in the nitrogen cycling of microbial communities [31]. The relative abundance of *Bradyrhizobium* was observed to be 1.8–3.7% in six treatments. After planting ryegrass, the relative abundance of *Bradyrhizobium* increased by 0.9% ($p < 0.05$), which was attributed to the special environment in the plant rhizosphere providing a habitat for nitrogen-fixing microorganisms [32]. As the level of CSA increased, the relative abundance of *Bradyrhizobium* decreased; the same trend was observed in Groups C and P, indicating that the impact of CSA on the relative abundance of Bradyrhizobium was negative. The highest relative abundance of Bradyrhizobium was observed in PT1, which may suggest that the role of CSA in nutrient cycling was not significant.

3.4. Functional Prediction

To further explore some functional genes closely related to promoting ecological functional construction in tailings by CSA and planting ryegrass, the potential functions of bacterial communities in all samples were predicted using KEGG pathways. Figure 3 shows the distribution of the top 20 functional genes (Level 2). Genes related to metabolism were dominant, with a relative abundance of 71.3% to 72.7% across the six treatments, which suggested that metabolism was the main activity of microorganisms in tailing soils. The rate of soil metabolic processes (respiration, digestion, denitrification, etc.) depends on the abundance of the related functional genes [33]. Toledo et al. [34] reported a significant correlation between the abundance of nutrient cycling genes and microbial diversity, in-

dicating that the relative abundance of functional genes can reflect the complexity of the microbial community's structure.

Furthermore, planting ryegrass increased the average relative abundance of metabolic functional genes by 0.4% (no significant difference). The slight increase in metabolism due to CSA in this study may be attributed to an increase in species rather than biodiversity. In the previous sections, we reported that CSA increased the Chao 1 index of soil (representing species richness) but decreased the Shannon and Simpson indices (representing species diversity). Planting ryegrass increased the quantity and diversity of microorganisms (Table S1), which can be attributed to the root environment providing organic matter. In the decomposition or humification of organic matter, microorganisms release energy for metabolism, leading to a further increase in the related functional genes [35]. The top three classes of metabolic functional genes with the highest relative abundance were amino acid metabolism (12.9–14.5%), carbohydrate metabolism (9.9–10.3%), and metabolism of cofactors and vitamins (9.0–11.5%). The functional genes, second only to metabolism in relative abundance, were related to processing genetic information, mainly including nucleotide metabolism; folding, sorting, and degradation; replication and repair; and translation functions.

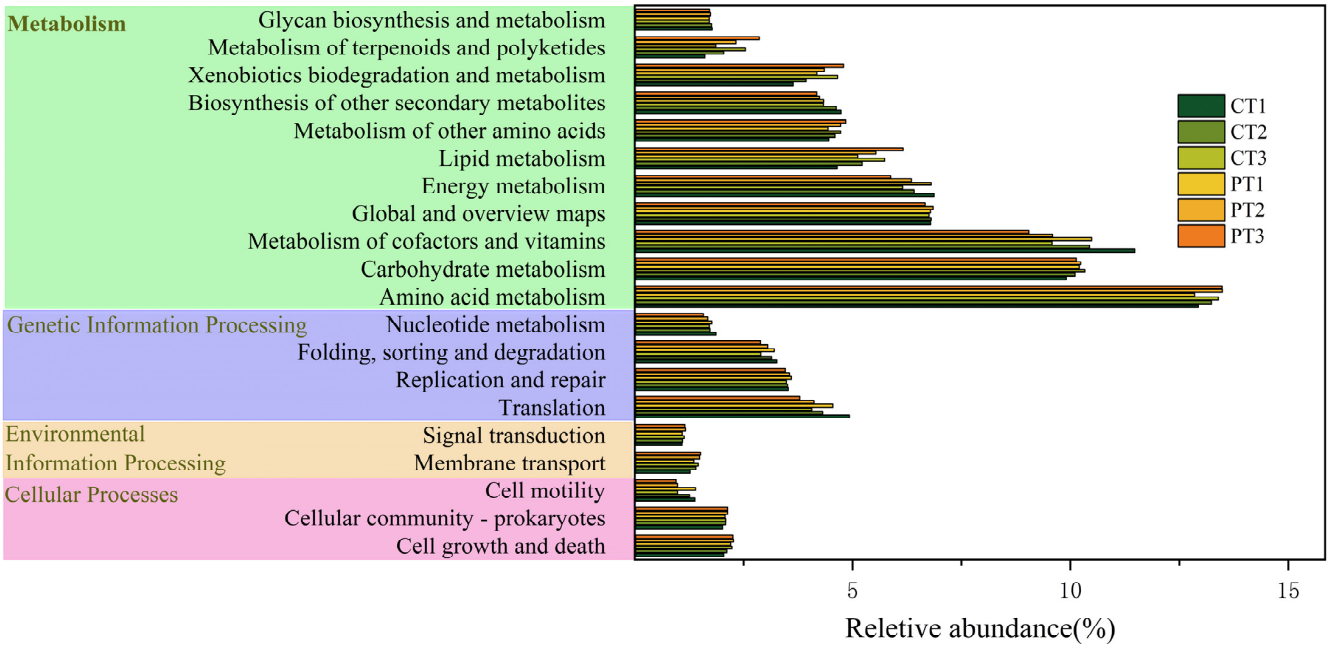


Figure 3. Variation in the profiles of bacterial function in tailing soils at KEGG Level 2.

3.5. Nitrogen and Cycling-Related Genes

3.5.1. Nitrogen Cycling-Related Genes

The abundance of genes related to cycling nitrogen and phosphorus was significantly correlated with soil nutrients (e.g., AN, AP) [36]. To investigate their correlation, the functions of genes in tailings were annotated according to the KEGG database. Figure 4 shows the relative abundance of the N cycling pathway genes in tailings. Specifically, the N cycling processes in tailings were divided into seven pathways: nitrogen fixation, nitrification, anammox, denitrification, dissimilatory nitrate reduction, assimilatory nitrate reduction, and ammonification.

From CT1 to CT3, with the increase in the proportion of clean soil, the relative abundance of ammonification and assimilatory nitrate reduction pathways decreased by 2.7% and 8.9%, respectively. The product of assimilatory nitrate reduction is amino acids, providing an N source for microorganisms; however, the addition of clean soil directly introduced an N source, thus leading to a decrease in this pathway. In contrast to assimilatory nitrate reduction, the relative abundance of the dissimilatory nitrate reduction pathway increased

from 19.1% to 23.8%. It is worth noting that the relative abundance of the nitrogen fixation pathway increased from 1.6% to 3.7%, an increase of 13.1%, which may be attributed to the role of nitrogen-fixing microorganisms present in the mature soil (clean soil). From the long-term perspective of remediating tailings, the environmental effect of increased AN directly caused by CSA was not as effective as enhancing the nitrogen-fixing function of tailings by inoculating them with microorganisms. The relative abundance of the denitrification and anaerobic ammonia oxidation pathways increased by 4.1% and 1.0%, respectively. Compared with Group C, the relative abundance of the assimilatory nitrate reduction, dissimilatory nitrate reduction, and nitrification pathways in group P decreased to varying degrees. Haichar et al. reported that plant root exudates inhibit soil nitrification processes, explaining the weaker soil nitrification in the plant treatment group [37]. The relative abundance of nitrogen fixation and denitrification increased from 2.3% and 23.0% to 5.2% and 24.8%, respectively, with increases of 126.1% and 7.8%, respectively. It is noteworthy that the relative abundance of the nitrogen fixation pathway reached 4.7% in PT1. This indicates that colonization by plants can significantly promote nitrogen fixation in tailings, because there are specific nitrogen-fixing microorganisms in the rhizosphere environment [38]. In addition, through a comparison of the abundance of nitrogen fixation pathways in six treatments, it was found that colonization by plants had a stronger promoting effect on nitrogen fixation in tailings than CSA, which implies that the effect of CSA may be overestimated. Figure 5 shows the changes in the level of nitrogen cycling genes, with a total of 22 nitrogen cycling-related genes detected. Three nitrogen fixation genes (nifH, nifD, and nifK) all showed an increasing trend with the proportion of clean soil, and their total abundance increased by 69.0% after colonization by plants. This change in the trend explains the changes in the relative abundance of the nitrogen fixation pathway. Three genes related to nitrification (pmoC-amoC, nxrB, and nxrA) were detected, and these genes were affected differently by the proportion of clean soil, but decreased after colonization by plants. Genes related to ammonia oxidation (nirK and nirS) and assimilatory nitrate reduction (nirA, narB, nirB, and nasA) showed the minimal influence of colonization by plants, with almost no significant difference in abundance before and after planting. The gene *norB*, which is responsible for denitrification, had the highest relative abundance, and both CSA and planting ryegrass could increase the relative abundance of norB.

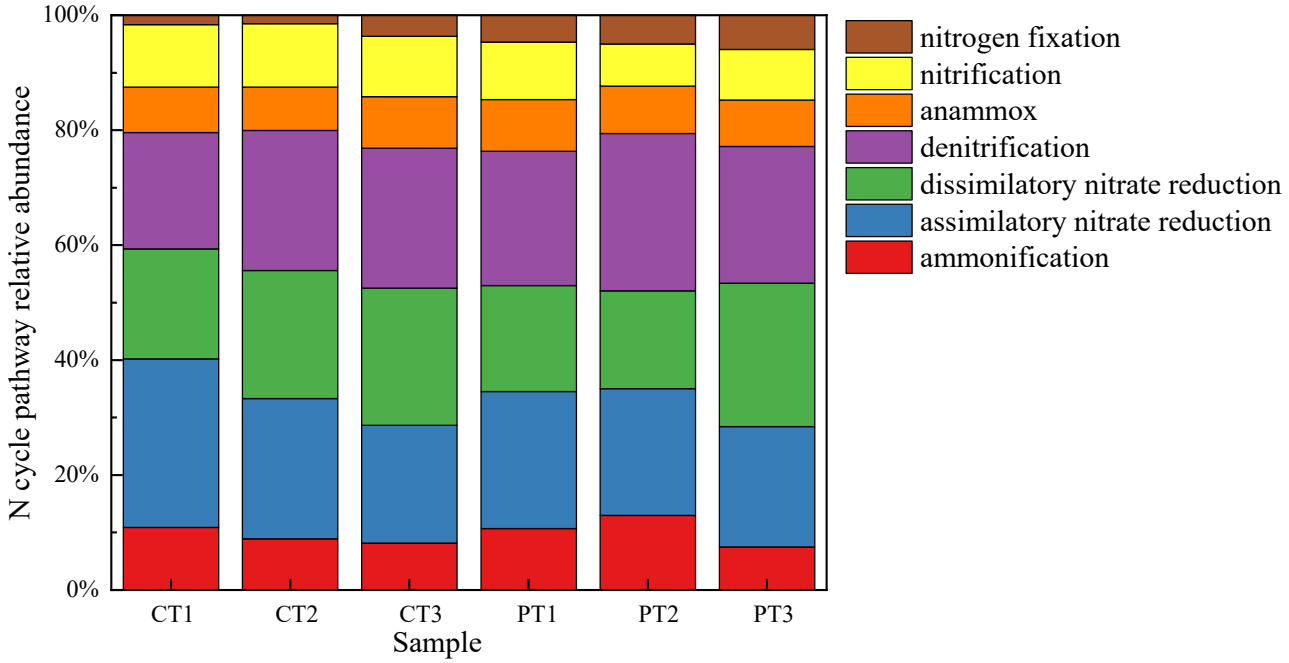


Figure 4. The relative abundance of the N cycling pathways.

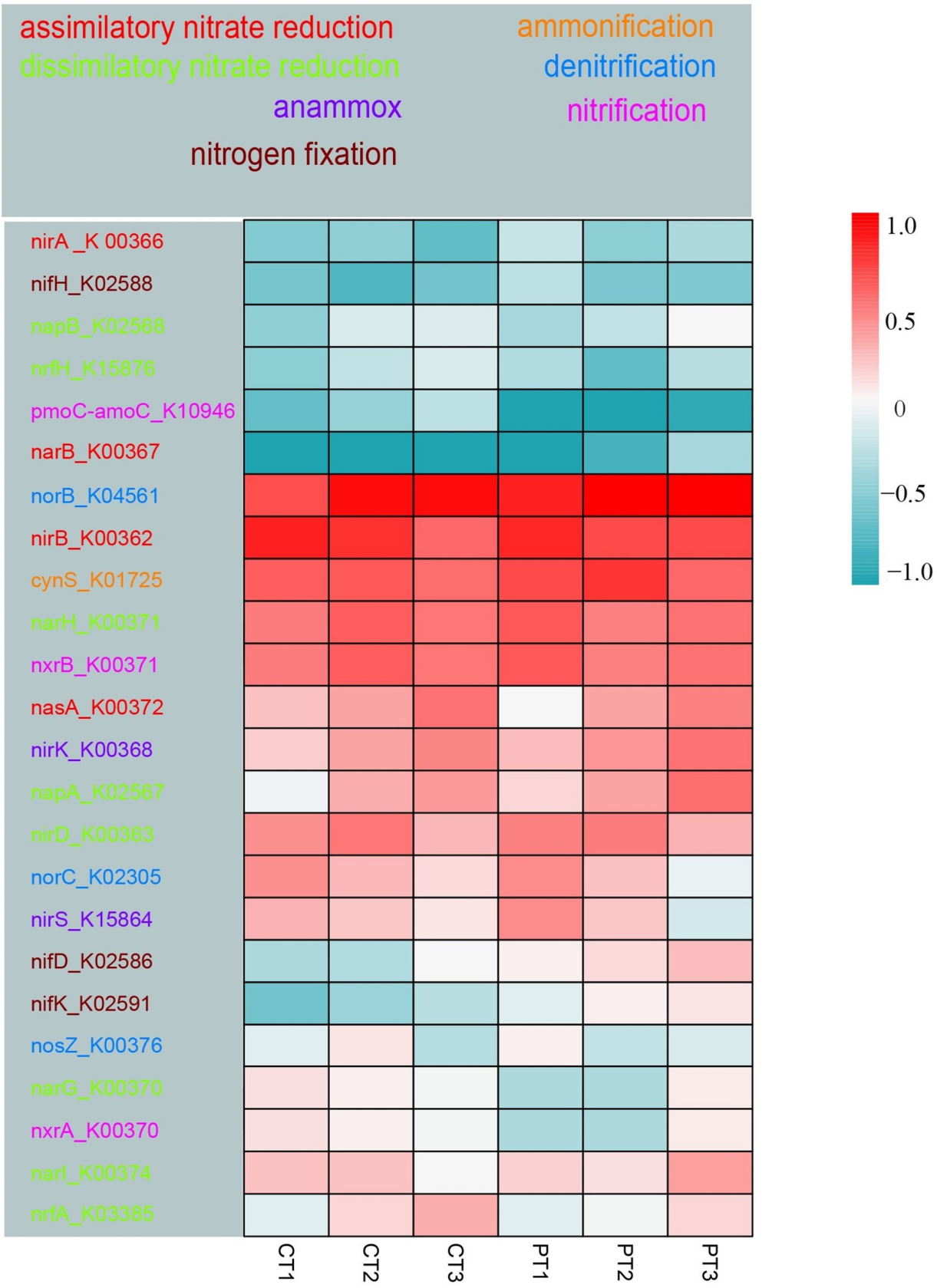


Figure 5. The relative abundance of N cycling-related genes in tailing soils. The corresponding value of the thermal map for each row is 10 times the genes' relative abundance.

3.5.2. Phosphorus Cycling-Related Genes

Figure 6 displays the relative abundance of phosphorus cycling pathways in tailings, with seven P cycling pathways detected, including phosphonate transportation, regulation of the P-starvation response, phosphate ester mineralization, inorganic phosphate transportation, inorganic phosphate solubilization, phosphate transportation, and phosphonate mineralization. Regulation of the P-starvation response, inorganic phosphate transportation, and inorganic phosphate solubilization were the predominant P cycling pathways in all samples, with relative abundances of 20.1–21.5%, 24.5–29.7%, and 20.9–32.2%, respectively.

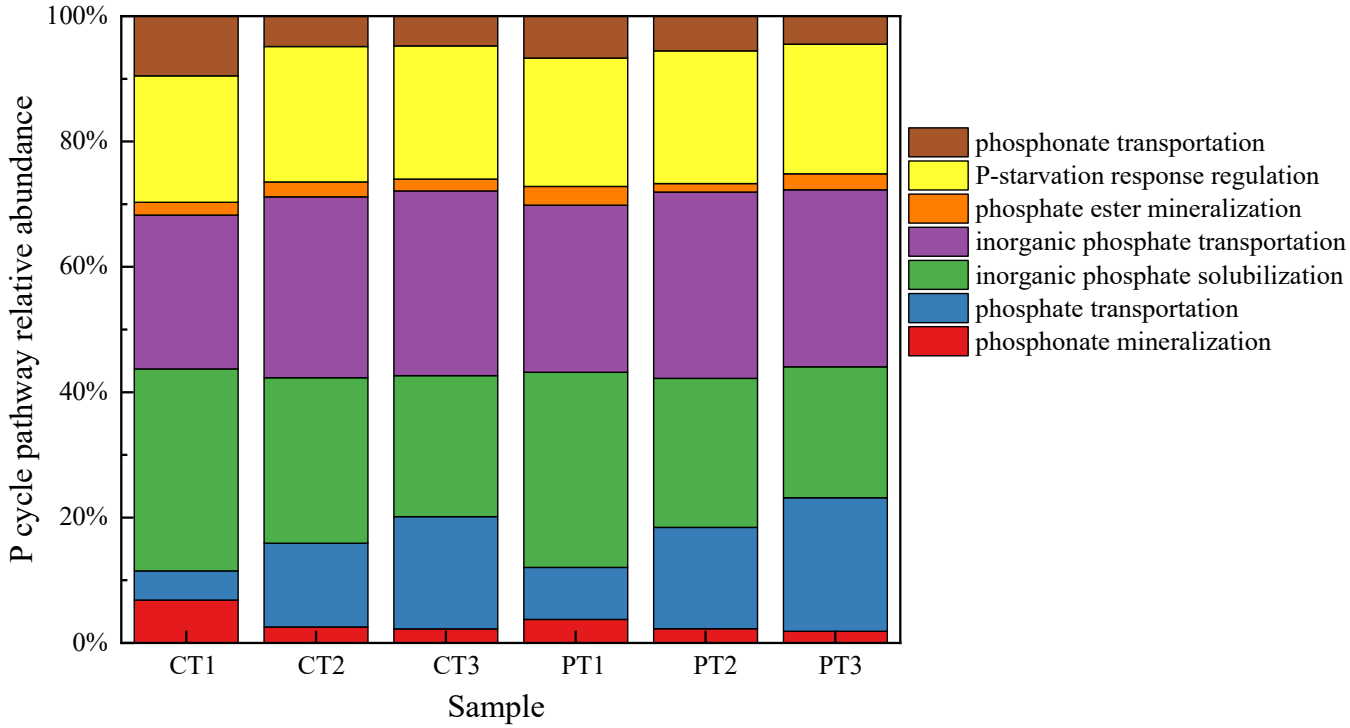


Figure 6. The relative abundance of the P cycling pathways.

Figure 7 shows the changes in the levels of phosphorus cycling genes. From CT1 to CT3, the relative abundance of the inorganic phosphate transportation and phosphate transportation pathways increased with the proportion of clean soil. The relative abundance of phosphate transportation increased from 4.6 to 17.9%, with an increase of 289.1%. CT1 had the highest abundance of genes related to phosphonate mineralization (phnL, phnJ, phnM, and phnI), and both CSA and planting ryegrass significantly reduced the abundance of these genes. Previous studies have reported that soilless restoration of vegetation in gold tailings also reduced the abundance of genes related to the mineralization of phosphonate [3]. Compared with CT1, higher abundances of genes related to phosphate transportation, inorganic phosphate solubilization, and inorganic phosphate transportation were observed. The increase in the abundance of these three genes explained the changes in AP. The *phoD* gene, as the most abundant gene in the alkaline phosphatase family of terrestrial ecosystems, plays an important role in the mineralization of organic phosphorus (Wang et al., 2021 [39]; Zhang and Lv, 2021 [38]). Therefore, organic matter from the sources of clean soil and organic matter secreted by plant roots can increase the abundance of the *phoD* gene.

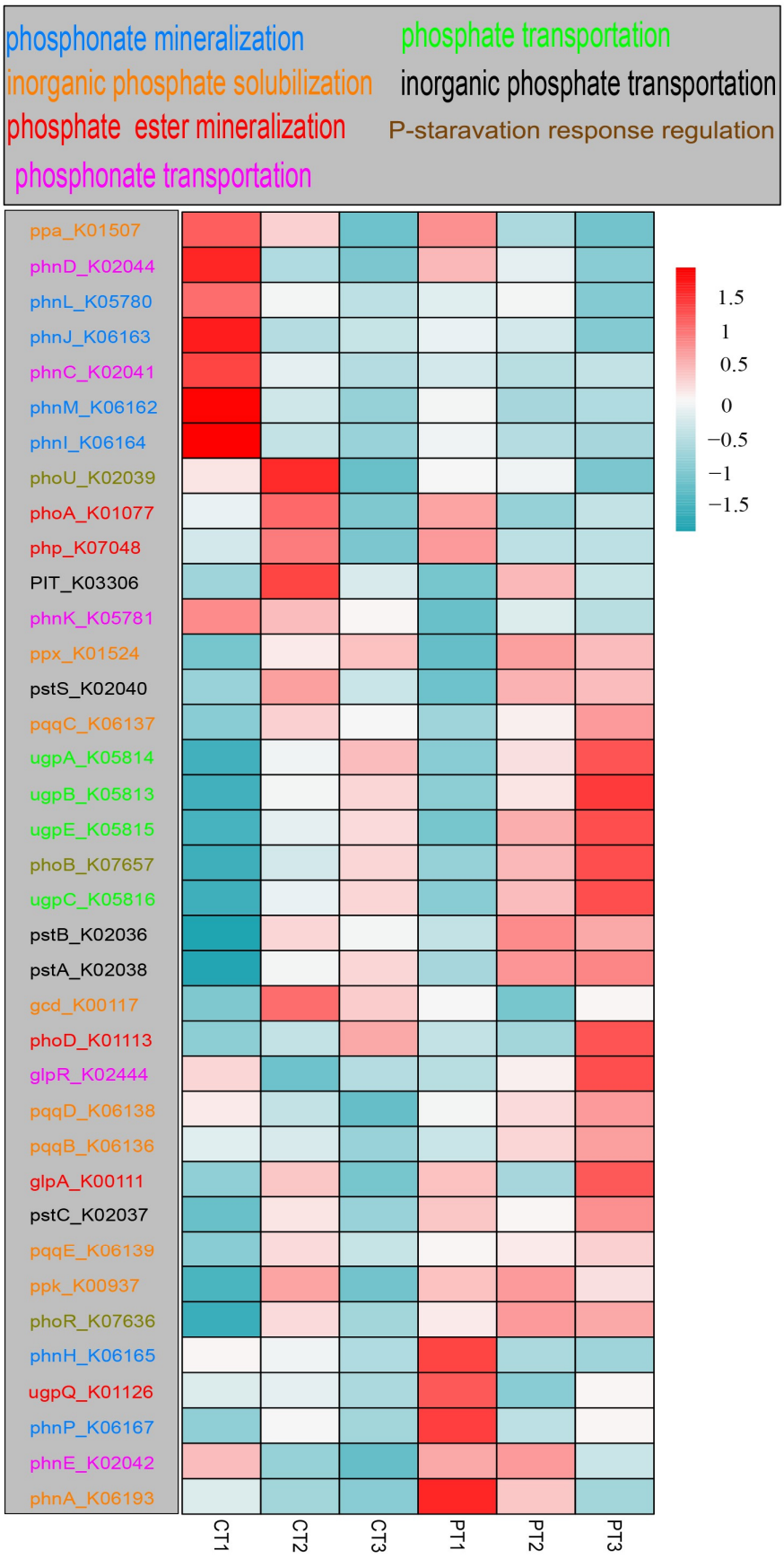


Figure 7. The relative abundance of P cycling-related genes in tailing soils. The corresponding value of the thermal map for each row is 10 times the genes' relative abundance.

4. Conclusions

In this study, using ryegrass as the selected plant, the phytoremediation of tungsten tailings was conducted under conditions of CSA. The research focused on investigating the variation in the microbial community's structures and elucidating the variation in the metabolic pathways and relative abundance of nutrient-cycling genes. The results suggest that CSA and planting ryegrass increased the microbial richness in tailings. CSA had a negative impact on the microbial community's evenness (Shannon index) and richness (Simpson index). In all treatments, the relative abundance of Pseudomonadota ranged from 64.4% to 75.2% and dominated the microbial community. High levels of CSA (T3) reduced the relative abundance of Pseudomonadota by 10–13%, and a higher relative abundance of Ascomycota was observed after planting ryegrass. At the genus level, the growth of ryegrass benefitted from the decrease in the abundance of Pseudomonas, Phenobacterum and Sphingobium after CSA. Cultivation of ryegrass increased the relative abundance of the nitrogen-fixing bacterium Bradyrhizobium (0.9%), which is beneficial for the sustainability of remediating tailing soils. Metabolism is the main activity of microorganisms in tailing soils, with a relative abundance of 71.3% to 72.7%. The changes in the abundance of genes related to nitrogen and phosphorus cycling indicated that CSA increased the nitrogen fixation function of microorganisms, with an increase of 131.5%. After planting ryegrass, the abundance of three nitrogen-fixing genes (nifH, nifD, and nifK) increased by 69.0%. Regulation of the P-starvation response, inorganic phosphate transportation, and inorganic phosphate solubilization were the predominant P cycling pathways, with relative abundances of 20.1–21.5%, 24.5–29.7%, and 20.9–32.2%, respectively. CSA and planting ryegrass significantly reduced the abundance of phosphonate mineralization-related genes (phnL, phnJ, phnM, and phnI).

Generally, the changes in microbial community's composition indicated that CSA and cultivation of ryegrass are beneficial for tailings. Still, the negative effects of CSA on microbial evenness (Shannon index) and richness (Simpson index) need attention. After remediation with the combination of CSA and planting ryegrass, the changes in N and P cycle-related genes were beneficial, such as the increase in the relative abundance of nitrogen-fixing genes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su16135715/s1, Figure S1: Total and bioavailable contents of (A) Cd, (B) W, and (C) Pb in the different treatments after 2 months of plant growth; Table S1: The property of the clean soil for addition; Table S2: The effect of CSA ratio on the growth of ryegrass; Table S3: The effect of SC level and ryegrass revegetation on tailings properties [40–43].

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