The microbial community composition and population change during bioremediation of uranium tailings

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Abstract. Uranium tailings produced in the process of uranium mining and metallurgy are an important source of uranium pollution, and pose a serious threat to the ecosystem and human health. In this study, Bacillus sp. as a functional bacterial when the uranium tailings were remediation in situ at 30°C and pH 6.5, aim to explore the response of the indigenous microbial community to environmental changes during the restoration process. The result indicted that after 14 days of remediation, the lowest uranium concentration obtained is 25.29mg/L. Next, 16s rRNA gene sequencing was used to reveal the dynamic changes of the microbial community structure during the competition process. Firmicutes and Proteobacteria are the two dominant phylum in the environment, the total highest contribution rate is 97%. Additionally, a significance present of sulfate-reducing bacteria such as Desulfotomaculum, Anaerocolumna, Burkholderia were detected in the U-treated microcosms comparison with repair initial. The results of this paper show that Bacillus sp. can be used as a functional bacteria to remediation the uranium tailings, and it is beneficial to the growth of functional microorganisms such as sulfate-reducing bacteria in the environment.

1. Introduction
The demand for uranium resources is increasing with the development of the nuclear industry, the process of uranium mining and metallurgy has caused amount of uranium tailings to accumulate in the tailings pond. Uranium commonly exists in the environment as either U(VI) or U(IV), however U(VI) more solubility and toxicity in oxidation state\textsuperscript{[1]}. Uranium has heavy metal toxicity and radioactive hazards, and long-term exposure to radioactive decay by-products of uranium may increase the incidence of cancer and other diseases. Therefore, the remediation of uranium tailings is of particular importance\textsuperscript{[2]}.

Traditional remediation methods (such as soil replacement and chemical leaching) destroy the physical and chemical properties of the soil and easily cause secondary pollution. However, bioremediation has the advantages of economy, environmental protection, simplicity and efficiency, so
gradually becomes a promising alternative method\cite{3}. To date, bioremediation methods mainly include bioreduction, biomineralization, biosorption, bioaccumulation, and so on. Among them, the precipitation product of uranyl-phosphate formed by the combination of phosphate and uranyl ions is very stable and difficult to oxidize\cite{4}. It has gradually become one of the more attractive remediation technologies to reduce the dissolution of harmful metals in tailings. Currently, *Citrobacter sp.*, *Bacillus sp.*, *Rahnella sp.*, *Pseudomonas sp.* have been used for biomineralization of uranium in acidic to neutral pH range.

Interestingly, *Bacillus sp.* have been found in many remediation areas, such as Oak Ridge, Meghalaya in India\cite{5}, as well as Rifle, Colorado\cite{6}. *Bacillus sp.* can remove uranium pollution in the environment through biological precipitation and biological adsorption, and there are literature reports that uranium stress may induce microbial phosphatase expression as a metal detoxification mechanism. Most studies have found that the microorganisms who used organic phosphate as the phosphorus source, can remove environment approximately 90% of uranium in environment, and the potential of naturally-occurring organophosphates used as a source of inorganic phosphorus to promote U(VI)-phosphate biomineralization has not been explored\cite{7}. In addition, a little information about the dynamic microbial changes during the bioenhancement process.

This study aims to identify the feasibility of the phosphate rock acted as a source of phosphorus, and *Bacillus sp.* is used to remediate uranium pollution in uranium tailings, thereby enhancing the utilization of phosphate minerals in the environment and reducing remediation costs. At the same time, high-throughput sequencing technology is used to sequence environmental samples at different periods to reveal the bacterial community diversity and community dynamics. So, this study will help to promote the understanding of the in-situ microbial community’s response to environmental changes and the reconstruction process after the introduction of exogenous microorganisms.

2. Materials and method

2.1. The Source of Bacteria and Medium

This strain (identified as *Bacillus sp.*) was initially isolated and purified from an uranium tailings pile in southern of China, and preserved in National Engineering Laboratory of Biohydrometallurgy, GRINM Group Corporation Limited.

The *Bacillus sp.* was cultivated in the LB liquid medium with 10g/L Tryptone, 5 g/L yeast extract, 10 g/L NaCl(pH=7), and incubated overnight at 30 °C with constant shaking(150 rpm), to observe the counts and activity of the bacteria, finally the resulting bacterial suspension with $1.0^8$ CFU/mL was reserve.

2.2. Uranium Tailings Remediation Experiment

Uranium tailings remediation experiment was carried out in a 1000-mL Erlenmeyer flask, and each containing 500g uranium tailings and 500mL NBRI P medium(Freshwater Bacteria Release Methane as a By-Product of Phosphorus Acquisition), which contained(g/L):NaCl(3), MgCl$_2$6H$_2$O(6), KCl(2.5), (NH$_4$)$_2$SO$_4$(0.1), Phosphate rock powder(5), and glucose(5). In this culture medium, the initial pH value of 6.5 and temperature of 30°C. In addition, the inoculum was 15%(v/v) of the bacterial suspension for *Bacillus sp.* inoculation. Samples of 15 mL were regularly taken to determine the uranium concentration, pH, and monitor the dynamic change of microbial community.

2.3. DNA Extraction, PCR Amplification and High-Throughput Sequencing

Microbial community genomic DNA was extracted from samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to manufacturer’s instructions. The DNA extraction was checked on 1% agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). The hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified with primer pairs 338F (5’-ACTCCTACGGAGGCAGCAG-3’) and 806R(5’-GACTACHVGGGTWTCTAAAT-3’) by an ABI
GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). The PCR amplification of 16S rRNA gene was performed as follows: initial denaturation at 95 °C for 3 min, followed by 27 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 10 min, and end at 4 °C. The PCR mixtures contain 5 × TransStart FastPfu buffer 4 μL, 2.5 mM dNTPs 2 μL, forward primer (5 μM) 0.8 μL, reverse primer (5 μM) 0.8 μL, TransStart FastPfu DNA Polymerase 0.4 μL, template DNA 10 ng, and finally ddH2O up to 20 μL. PCR reactions were performed in triplicate. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to manufacturer’s instructions and quantified using Quantus™ Fluorometer (Promega, USA).

Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The data were analyzed on the free online platform of Majorbio Cloud Platform (www.majorbio.com).

2.4. Analytical Methods
Cell extracts were filtered using a 0.22 μm filter system before U concentration and pH were measured, the total U concentration was determined by ICP-MS (Agilent Technologies 7700x, USA). The pH of was measured using a pH meter (STARTER 3100, Ohaus Corporation, Shanghai, China).

3. Results and Discussion

3.1. Removal performance of uranium
The uranium concentration and pH changes during the remediation process are shown in Figure 1. Within 7 days, a large amount of uranium in the tailings is dissolved out. The uranium concentration in the environment increases with the increased of the cultivation time, and the highest content exceeds 100 mg/L. From 7 to 21 day, the uranium concentration is decreased and the lowest uranium concentration is 25.29 mg/L obtained in 14 days. However, there was a negative correlation between pH and uranium concentration. Within 7 days, the pH show a downward trend of 5.43, which is conducive to the dissolution of uranium in the environment, and then show slight fluctuations without significant changes.

![Figure 1. The uranium concentration and pH value in uranium tailing remediation by Bacillus sp. during different period.](image)

Bacillus sp. can effectively remediation uranium pollution in uranium tailings and reduce the dissolution of uranium from tailings. Beazley et al. [8] founded that Bacillus sp. isolated from FRC soils can remove 73-95% of total uranium in simulated groundwater. And Huang et al. [9] reported that U(VI) was precipitated by Bacillus and produce a hydrogen uranyl phosphate involved in the uranium phosphate mineral precipitation. In this study, the lowest uranium concentration, which fully meets the uranium concentration standard in groundwater (<30 μg/L) in the environment. Based on this result, the
introduction of *Bacillus sp.* using phosphate rock as a source of phosphorus can reduce the dissolution of uranium from uranium tailings to a certain extent.

### 3.2. Microbial richness and diversity changes

During the culture time, microorganism alpha indexes and coverage are shown in Table 1. The coverage of all sample is above 0.995, proving that most of the sequences contained in the uranium tailing were detected. Alpha indexes can reflect the richness and diversity of microorganisms, Shannon and Simpson indexes are used to reflect microbial diversity, while ACE and Chao indexes are used to evaluate richness \[^{[10]}\]. In this study, the increase of Shannon index and the decrease of Simpson index is positively correlated with time, indicating that microbial diversity obviously increases with time increases.

**Table 1. Alpha index of bacterial diversity during the different period of remediation.**

| Days | Shannon | Simpson | ACE   | Chao   | Coverage |
|------|---------|---------|-------|--------|----------|
| 1    | 1.39    | 0.31    | 427.33| 256.02 | 0.999    |
| 7    | 2.52    | 0.16    | 255.98| 226.48 | 0.999    |
| 14   | 3.46    | 0.09    | 310.85| 308.68 | 0.999    |
| 21   | 2.46    | 0.19    | 331.39| 263.73 | 0.999    |

After 14 days, Shannon grows to the maximum value time, up to 3.46, while Simpson reaches to the minimum about 0.086. Which indicted that the diversity of microorganisms reached the maximum. On contrary, ACE and Chao show a positive correlation with time, get the maximum value in 21d, the value are 331.39 and 263.73, it shows that prolonging the culture time is beneficial to increase the abundance of microorganisms.

**Figure 2. The Venn diagram in uranium tailing remediation by *Bacillus sp.* during different period (LK1-1 day, LK2-7 day, LK3-14 day, LK4-21 day).**

The Venn diagram can be used to count the number of common and unique species (such as OTU) in multiple groups or samples, and can more intuitively show the similarity and overlap of species (such as OTU) in different environmental samples. The Venn diagram during the remediation process is shown in Figure 2. With the extension of the culture time, the microbial richness content in the environment is LK3>LK4>LK2>LK1. The number of unique species in different remediation periods showed LK3>LK1>LK4>LK2. Combined with the analysis of Figure 1, it shows that the abundance of microorganisms in the environment is the highest at 14d of remediation, and the microorganisms that can remove uranium are among them.

### 3.3. Change and reconstruction of microbial community

In order to reflect the process of microbial community competition and reconstruction, the analysis results at different periods(at the phyla level) are shown in Figure 3. The results indicate that the main
microbial phyla in the tailings remediation environment is Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes, with a cumulative contribution was above 99%.

Firmicutes and Proteobacteria are the two dominant phylm in the environment, the highest content of the two is more than 97% obtained in 21d. The Firmicutes phyla was previously reported. Ding et al stimulated indigenous microorganism by adding carbon source into in situ leaching uranium mine, then found that Firmicutes(>70%) was dominant in the sediment[11]. For Proteobacteria, it has been is applied in uranium remediation for uranium-contaminated soil during the composting process[12].

It can be seen from Figure 3, Actinobacteria dropped from the initial 67.62% to 1.21%, but there is no report on the reasons for the decrease. Therefore, it is speculated that Actinobacteria is at a disadvantage in the process of bio-enhanced remediation, and the competition with other species of organisms resulting in a significant decrease in content. Interestingly, Bacteroidetes reached to a maximum value of 1.7% at 14d with the cultivation time. Martinez et al[13] reported that Bacteroidetes can promote the biomineralization of uranium during organophosphate amendments, which indicated that organophosphate could promote the Bacteroidetes growth.

Figure 4 shows the change process of the microbial communities at the genus level. With the extension of the remediation time, the structure and abundance of the dominant genus in the environment have undergone major changes. In initial, the main microorganisms are composed of Rhodococcus(22.89%), Paenarthrobacter(18.69%), Pseudomonas(8.89%), Bacillus(17.7%), Arthrobacter(24.11%). From 1d to 7d, the abundance of Pseudomonas, Clostridium sensu stricto, Azotobacter was shown a significant growth, they were 43.77, 11.9 and 22.41. On the contrary, Rhodococcus, Paenarthrobacter and Arthrobacter in the original environment dropped sharply and remained at a very low level after 7 days. Related reports pointed out that Clostridium sensu stricto, Azotobacter and Pseudomonas promoted uranium leaching from tailings. The results show that Bacillus sp. can inhibit the growth of Rhodococcus, Paenarthrobacter and other organisms.

From 7 to 21 days, Clostridium sensu stricto and Pseudomonas decreased 50%, however, Desulfotomaculum, Anaerocolumna, Burkholderia which were U(VI)- reducing bacteria found in the uranium remediation composting system, showing a growing trend and gradually transforms into a dominant genus, up to the highest abundance is 8.45%, 22.37% and 3.21% at 14d. Desulfotomaculum was the typical sulfate- and metal- reducing bacterium, which can be efficiently used for uranium pollution treatment and bioremediation. At the same time, Anaerocolumna is a kind of anaerobic bacteria, which was previously found in uranium-containing environment, which can attribute to U(VI)-reducing effectively[14].

Based on the above analysis, the bioenhanced restoration of uranium tailings by introducing Bacillus sp. can effectively stimulate the growth of indigenous microorganisms, such as
Desulfotomaculum and Anaerocolumna, thereby reducing uranium in the environment and providing a good remediation environment for indigenous microorganisms.

4. Conclusion
The present study reveals reveals the feasibility of phosphate rock used as the phosphorus source for Bacillus sp., and finds that the biomineralization of uranium tailings can effectively reduce the dissolution of uranium in the tailings. The best remediation effect appears at 14d, and the uranium concentration is 25.29 μg/L. The introduction of exogenous microorganisms reduces the uranium in the environment, which is beneficial to increase the diversity and abundance of microorganisms in the environment, and the diversity and abundance of microorganisms appears when remediation effect is best. Furthermore, a significant change of Firmicutes and Proteobacteria phyla is observed in comparison with the initial. The abundances of the Rhodococcus, Paenarthrobacter and Arthrobacter have shown a sharp decline. As the concentration of uranium in the environment increases, it shows that they are not adapted to the uranium environment. However, Desulfotomaculum, Anaerocolumna, Burkholderia, Clostridium_sensu_stricto and Pseudomonas show a corresponding growth, with the uranium concentration is decreasing, suggesting they contributs to uranium removal. A greater degree of dynamic change of bacterial communication and abundance was observed during the period when the best bioremediation effect was observed.

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