Radioprotection 2019, 54(3), 219–224

https://doi.org/10.1051/radiopro/2019026

Available online at:
www.radioprotection.org

ARTICLE

Effects of thorium on paddy soil enzymes and microbial diversity

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Received: 1 August 2018 / Accepted: 19 June 2019

Abstract – We conducted an experiment to investigate the effects of ^{232}Th on soil enzymes and microbial diversity in soils. Under each treatment, elevated ^{232}Th obviously inhibited the activity of soil enzymes such as urease (UR), dehydrogenase (DH), catalase (CAT), phosphatase (PHO) and aryl sulfatase (AS). In each treatment, *Proteobacteria* was the most dominant flora followed by *Actinobacteria* and *Acidobacteria*. *Pseudomonas sp.* was the dominant strain. This study might provide the preliminary analysis of soil enzymes and microbial diversity in Th contaminated soils.

Keywords: thorium / soil enzymes / microbial diversity / paddy soils

1 Introduction

Soil is an important resource in the agricultural production, particularly in the Northeast of China (Qiu *et al.*, 2005). Human-induced soil environmental change can be as an indicator of soil resources and environmental quality, and it is related to global changes and sustainable development (Hu *et al.*, 2017). With the rapid development of the nuclear industry and nuclear energy, the demand for uranium mining metallurgy products keeps increasing. However, extraction of uranium and ore proceeding in milling facilities produce large solid wastes (uranium mill tailings) which contain a series of long-lived radionuclides such as thorium (^{232}Th) which belongs to the family of high toxic radionuclides. So if the uranium mill tailings were discharged into the environment, the soil will be polluted severely.

Soil enzyme activities have been suggested as suitable indicators for soil quality, which could be used to measure the degree of soil degradation and the impact of soil pollution (Gianfreda *et al.*, 2005). DH, AS and CAT are reported to be associated with microbial activity, while UR and PHO play important roles in N, P and S cycles. In addition, soil enzymes activities are found to be significantly correlated with soil pH (Acosta-Martinez and Tabatabai, 2000). Also there is strong evidence that soil microbe diversity were sensitive to radionuclide contamination (Yan and Luo, 2015). However, the effect of ^{232}Th on soil enzymes and microbial diversity is less known. Thus we conducted experiments by using soil samples collected from Northeast of China with different thorium concentrations (two experimental groups and control group). The specific objectives of our experiments were to examine (1) the effects of different concentrations of ^{232}Th on soil enzyme activities such as urease (UR), dehydrogenase (DH), catalase (CAT), phosphatase (PHO) and aryl sulfatase (AS) and (2) the effects of different concentrations of ^{232}Th on soil microbial diversity by denatured gradient gel electrophoresis technology (DGGE).

2 Methods and materials

2.1 Soil and plants for the experiment

Soil samples for the experiment were collected from farmland soils cultivated with corn, situated in the Northeast of China. They were clayey soil, classified as an Ultisol in US soil taxonomy, with 70.15% clay, 10.22% silt and 16.78% sand. The soil had 20.39 g kg^{-1} organic matter, 125.7 mg kg^{-1} available nitrogen, 12.5 mg kg^{-1} available phosphorus, 80.7 mg kg^{-1} exchangeable potassium, 211.2 mg kg^{-1} exchangeable calcium, 37.2 mg kg^{-1} exchangeable magnesium. Radionuclide ^{232}Th concentration in soil sample was 12.8 mg kg^{-1}. Collected soil samples were packed into a water-tight bag to prevent cross-contamination and shipped to the laboratory.

2.2 Experimental design

Usually the uranium mill tailings were deposited in a specially designed and constructed repository. However they can be removed from uranium tailings for a variety of reasons such as wind erosion, rain wash and the damage of soil cover.

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Then it will lead to different levels of Th in soils. So in the present study, three levels of ^{232}Th 0 mg kg^{-1} (T1, control), 60 mg kg^{-1} (T2) and 100 mg kg^{-1} (T3) were added to simulate ^{232}Th pollution sites around uranium tailings. Firstly ^{232}Th was added to the mesocosms as thorium oxide (ThO_2). And then the mesocosms were filled with contaminated soils and mix thoroughly together. Eighteen mesocosms (50 × 50 × 20 cm) were used in this experiment (6 mesocosms in each treatment). In other words, there are 6 mesocosms in each treatment. Each mesocosm was filled with 7 kg yellow soils. Then mesocosms were arranged in greenhouse in a complete randomized block design. Onemonth after ^{232}Th addition, 6 soil samples were collected from each ^{232}Th treatment to determine the total ^{232}Th concentrations using inductively coupled plasma mass spectrometry (modal – NexION 300Q). The detection limit was 0.0003 μg/L for ^{232}Th. The accuracies of inductively coupled plasma mass spectrometry analyses are estimated to be better than ±5% (relative) for the elements determined. The total concentrations of the 0, 60 and 100 mg kg^{-1} ^{232}Th treatments were 13.25, 74.03 and 119.71 mg kg^{-1}, respectively.

2.3 Measurements

Eight months after ^{232}Th addition, soil samples were collected. For each treatment, six soil samples were collected at a depth of 0–15 cm and then combined (about 95 g). All soil samples were immediately transported to the laboratory and stored at 4 °C for analyzing the change of soil enzyme activity and microbial diversity. Soil enzyme activities, such as UR, DH and PHO were determined as described by Jyot *et al.* (2015), CAT was determined as described by Ma *et al.* (2015) and AS was determined as described by Rana *et al.* (2015).

For each soil sample, gDNA was extracted using sludge genomic DNA rapidly extraction kit (MP Biomedicals FastDNA). PCR amplification of bacterial 16S rDNA from gDNA was performed using the universal primers of GC-338F (with 40 GC clamps) and 518R. The PCR reactions were carried out in 50 μL volumes, containing 2 ng gDNA, 1 μL GC-338F (20 mM) and 1 μL 518R (20 mM), 5 μL 10 × PCR buffer, 3.2 μL dNTP (2.5 mM), 0.4 μL rTaq (5 U/μL), added to 50 μL with ddH_2O. Cycling conditions involved an initial 5 min denaturing step at 95 °C, followed by 30 cycles of 1 min at 95 °C, 50 s at 60 °C, and 1 min at 70 °C, and a final extension step of 10 min at 70 °C. After verification of PCR product in agarose gel, 10 μL PCR products were analyzed *via* DGGE. The deformation gradient was from 35 to 55% (100% denaturant corresponds to 7 mol/L urea and 40% (v/v) acrylamide) and the 8% polyacrylamide gel was electrophoresed in 0.5 × TAE running buffer for 5 h at 160 V and 50 °C. After that, the gel was detected by silver staining and pictured using BioRAD system. The specific bands in DGGE were recycled and amplified as a template. The primer sequences were 5'-ACTCCTACGGGAGGCAGCAG-3' (338f) and 5'-ATF ACCGCGGCT GC TGG-3' (518r). PCR products were sequenced. Sequencing reactions for PCR products were optimized using the BigDye Terminator v1.1 sequencing Ready Reaction kit (Applied Biosystems, CA, USA). The reactions system was comprised of 1 μl of purified PCR products, 1 μl of primer (3.2 pM/μl), 8 μl of 2.5 × BigDye Terminator enzyme, and 4 μl of 5 × sequencing buffer, and the reactions were started with an initial denaturation at 95 °C for 1 min, followed by 25 cycles of 95 °C/10 s, 50 °C/5 s and 65 °C/4 min. Next, the sequencing products were precipitated and sequenced in both directions *via* a Genetic Analyzer 3130*xl* (Applied Biosystems, CA, USA). Finally, the bands were checked using the BLAST search program at NCBI (National Centre for Biotechnology Information) (Yan and Luo, 2015). Sequences showing highest homology with the sequences in GenBank were selected. Furthermore, the phylogenetic characteristics for the soil samples were determined for mixed soil samples, briefly, the sequences of the microorganisms were transformed into the FASTA form, and then were checked using the Clustal-X software (Gao *et al.*, 2014). Neighbor–Joining was used to calculate the evolutionary distance, followed by the phylogenetic tree construction *via* MEGA V4.1, in combination with the Boot-straps test (Sun *et al.*, 2015). The diversity indexes were also calculated from the DGGE banding patterns to indicate the bacterial diversity of soil samples, including Shannon–Wiener Index, diversity of communities; Evenness, the degree of uniformity; Richness, the species numbers identified in a sample (Zhong *et al.*, 2014).

2.4 Data analysis

Multivariate analysis and analyses of variance (ANOVA) were used to demonstrate differences among the sample using SPSS 19.0.

3 Results and discussion

3.1 Soil enzyme activities

In the present study, the activities of all soil enzymes decreased with the increasing of ^{232}Th concentration and significant effects of ^{232}Th on soil enzymes activities were observed ($p < 0.05$) (Tab. 1). In other studies, soil enzyme activity has also been used to assess the influence of radionuclide pollution on soil quality. Zhang reported that radionuclide has inhibiting effects on all the four kinds of soil enzymes (Zhang *et al.*, 2015). Among these enzymes, activities of urease, phosphatase and sucrose decrease with the increase of the concentration of ^{232}Th. Xun collected soil samples from uranium mill tailing and also found that enzyme activities were all increased along with decreased radionuclide concentration (Yan and Luo, 2015).

Radionuclide inhibits soil enzymes activities in two ways. First, radionuclide can fit in the active sites (SH group and/or imidazole ligand) of enzyme molecule to form a stable complex compound, which was competitive inhibition. Second, radionuclide can combine with SH, NH_2, OH and COOH group to form strong covalent bond, and then result in the deformation of the three-dimensional structure of the

Table 1. Analysis of enzyme activities in soil samples (mean ± SD).

Treatment	UR	DH	CAT	PHO	AS
T1	$11.37^{a} \pm 0.66$	$173.1^{a} \pm 4.30$	$3084.9^{a} \pm 47.9$	$212.9^{a} \pm 6.97$	$127.6^{a} \pm 3.80$
T2	$6.58^{b} \pm 0.36$	$91.6^{b} \pm 2.69$	$1826.1^{b} \pm 48.5$	$143.0^{b} \pm 3.97$	$73.7^{b} \pm 2.83$
T3	$2.41^{c} \pm 0.25$	$33.7^{c} \pm 3.30$	$453.1^{c} \pm 6.99$	$94.6^{c} \pm 2.44$	$43.9^{c} \pm 2.90$

Different letters (a–c) indicate significant difference ($P < 0.05$).

enzyme (Xian *et al.*, 2015). Based on the above analysis, soil enzymes can be sensitive to reflect changes in soil quality which was contaminated by ^{232}Th.

3.2 Soil microbial diversity

Soil microbial diversity was analyzed *via* denaturing gradient gel electrophoresis (DGGE) technology. The diversity indexes such as Shannon–Wiener index (Si), Evenness index (Ei) and Richness index (Ri) were calculated (Tab. 2). The average values of Si which reflected the diversity of communities in each treatment were 2.16 ± 0.24 (T1), 1.93 ± 0.33 (T2) and 1.75 ± 0.31 (T3) respectively. The average values of Ei which reflected the degree of uniformity were 0.90 ± 0.06 (T1), 0.92 ± 0.02 (T2) and 0.87 ± 0.05 (T3) respectively. And the average values of Ri which reflected the species numbers identified in soil sample were 11.5 ± 2.66 (T1), 8.50 ± 2.74 (T2) and 7.50 ± 1.88 (T3) respectively. These results showed that the average values of Si and Ri decreased with the concentration of ^{232}Th increasing which indicated ^{232}Th have significant effect on Si and Ri in soils. Also we can see that there were differences of microbial diversity in different treatment block.

Total 9 bands (T1), 8 bands and 6 bands were sequenced and further conducted similarity sequence searching *via* Basic Local Alignment Search Tool (BLAST) in GenBank to detect the representative microorganisms (Fig. 1). For T1 block, four strains such as Proteobacteria, unknown phylum from environmental samples (the exact bacteria name could not be identified, as there is no detailed information of similar sequence related with the 'unknown environmental samples' in NCBI), Actinobacteria, and Firmicutes, were identified (Tab. 3 and Fig. 2). For T2 block, four strains such as Proteobacteria, Firmicutes, Actinobacteria, and Acidobacteria were identified (Tab. 3 and Fig. 2). For T3 block, two strains such as Proteobacteria and Acidobacteria were identified (Tab. 3 and Fig. 2). In Figure 2 we can see that, bands 6 were common between A and B treatments. However bands 11 common between B and C treatments. Similarly, bands 15 were common between B and C treatments. We can also see that bands 4 were present in C treatments. In present study, we can find that the diversity of microorganisms in all the soil samples is humdrum. In each treatment, Proteobacteria assumed absolute superiority and *Pseudomonas* sp. of Proteobacteria was the dominant strain.

Table 2. Analysis of diversity indexes from the DGGE banding patterns.

Samples	Si	Ei	Ri
T1	2.09	0.84	12
	2.49	0.92	15
	1.94	0.81	11
	2.28	0.89	13
	2.29	0.96	11
T2	1.86	0.96	7
	1.89	0.91	8
	2.39	0.93	13
	2.15	0.94	10
	1.95	0.94	8
	1.44	0.90	5
	1.75	0.90	7
T3	1.94	0.93	8
	1.94	0.88	9
	2.10	0.91	10
	1.69	0.87	7
	1.53	0.85	6
	1.28	0.79	5

Other studies have found similar results. Yan found that Proteobacteria was the most dominant flora according to GenBank database in uranium mill tailing soils (Yan and Luo, 2015). In Rastogi's study, the PhyloChip detected the largest number of taxa in Proteobacteria phylum for the uranium mine sites (Rastogi *et al.*, 2010). The abundance and composition of bacteria of the phylum Acidobacteria were surveyed in subsurface sediments from uranium-contaminated sites using amplification of 16S rRNA genes (Akob *et al.*, 2008). The 16S rDNA analyses showed that the uranium wastes were dominated by acidithiobacillus and several Pseudomonas species classified in the γ-subdivision of the Proteobacteria (Selenska-Pobell *et al.*, 2001). Based on these analyses, we can see radiation does not always inhibited microorganism growth. In present study, Proteobacteria phylum can be adaptable to the soils contaminated by ^{232}Th.

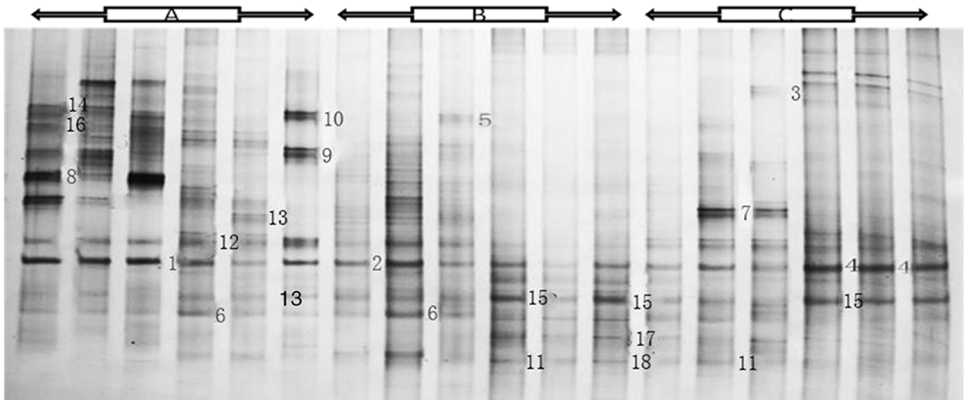

Fig. 1. The denaturing gradient gel electrophoresis (DGGE) profiles of amplified 16S rDNA fragments from soil samples used to for sequencing. A, B and C represented soil samples collected from T1, T2 and T3 block.

Table 3. Sequence comparative analysis of DGGE bands.

Samples	The most similar strains	Accession number	Similarity (%)	Phylum
Band1	Pseudomonas syringae	HF566316	100	Proteobacteria
Band2	Pseudomonas sp. pcs20	AY303244	100	Proteobacteria
Band3	Roseomonas sp. HPCKc-25	HE802768	89	Proteobacteria
Band4	Pseudomonas fluorescens	JQ974027	100	Proteobacteria
	Pseudomonas syringae	HF566316	100	Proteobacteria
	Pseudomonas sp. PSFX-1	JX885684	100	Proteobacteria
Band5	Firmicutes bacterium WSF2-15	FJ405898	94	Firmicutes
Band6	Arthrobacter sp. WS20	JN899573	99	Actinobacteria
Band7	Rhodanobacter sp. BJC16-A24	JX483760	99	Proteobacteria
	Dyella japonica	AM086249	99	Proteobacteria
Band8	Acidiferrobacter thiooxydans	AF387301	95	Proteobacteria
	Acidithiobacillus ferrooxidans	M79416	94	Proteobacteria
Band9	Actinobacterium BGR 43	GU167988	98	Actinobacteria
Band10	Agricultural soil bacterium CRS5633T18-1	HQ132677	96	Environmental samples
Band11	Acidobacteria bacterium AB158	JX029998	91	Acidobacteria
Band12	Gluconacetobacter xylinus	JQ513852	100	Proteobacteria
	Rhodopila globiformis	M59066	100	Proteobacteria
Band13	Rhodopila globiformis	NR_037120	100	Proteobacteria
Band14	Alicyclobacillus sp. CCUG 53762	HE613268	90	Firmicutes
Band15	Acidiphilium sp. MS8	AB561883	99	Proteobacteria
Band16	Bradyrhizobium jicamae	JX469395	100	Proteobacteria
	Rhodopseudomonas rhenobacensis	JX282403	100	Proteobacteria
Band17	Novosphingobium sp. FNE08-86	JN399173	99	Proteobacteria
	Erythrobacter sp. H504	HQ622548	99	Proteobacteria
Band18	Acidobacteriaceae bacterium MCF14	JX412364	99	Acidobacteria

4 Conclusions

In conclusion, different ^{232}Th concentrations used in this study significantly affect soil enzyme activities and microbial diversity. This present study might provide the preliminary analysis of soil enzymes and microbial diversity in radionuclide ^{232}Th contaminated soils in China.

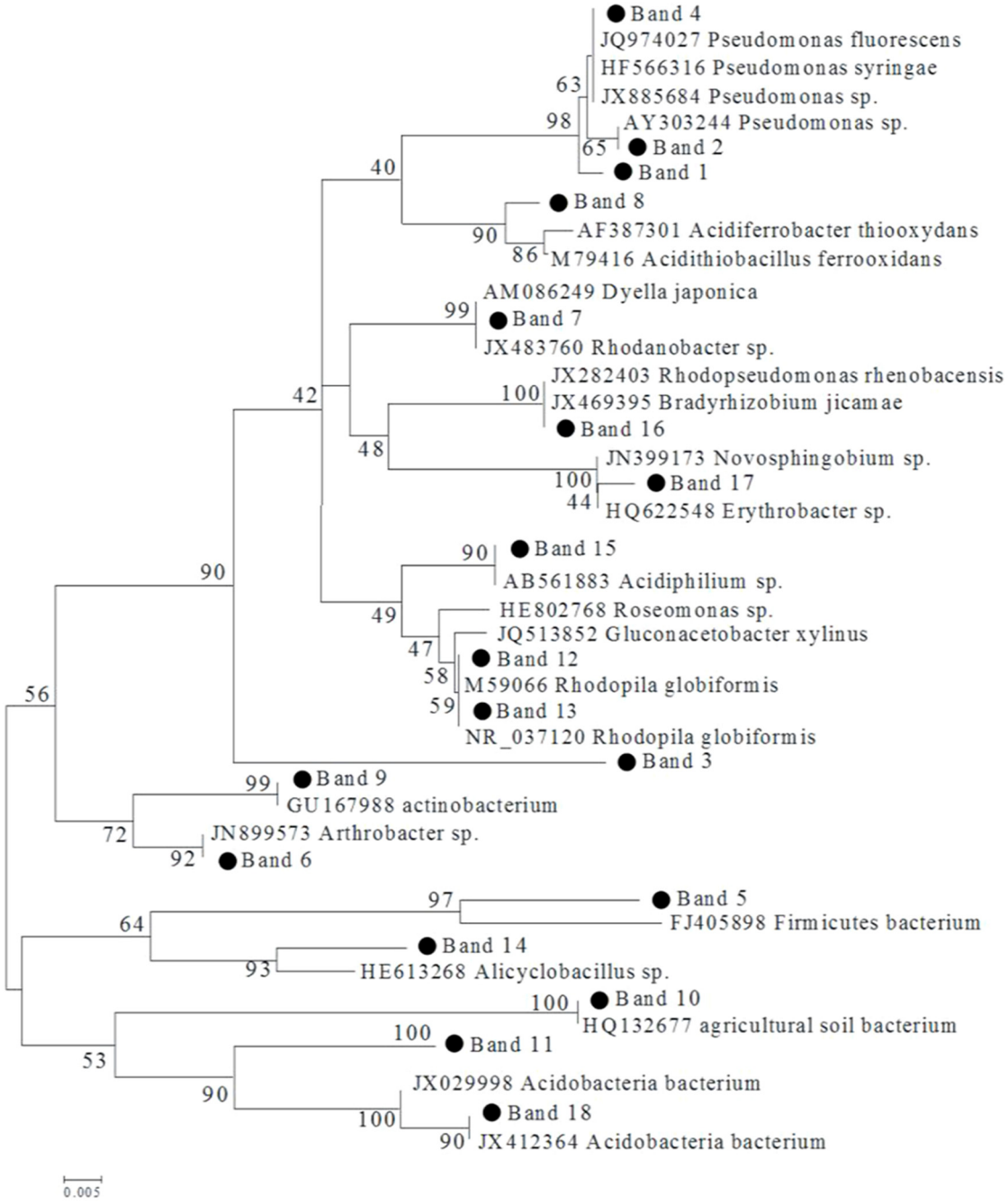

Fig. 2. Phylogenetic analysis of 16S rDNA gene sequences from gDNA.

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Cite this article as: Zhu Z. 2019. Effects of thorium on paddy soil enzymes and microbial diversity. *Radioprotection* 54(3): 219–224